IDENTIFICATION OF IMPORTANT AMINO ACID RESIDUES IN THE ACTIVE SITE OF YEAST F_1-ATPASE AND IN THE BEEF HEART F_1-ATPASE INHIBITOR PROTEIN

By

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ABBREVIATIONS

ADP, adenosine-5'-diphosphate

amino acids are abbreviated according to the one letter code and the three letter code

ATP, adenosine-5'-triphosphate

BCA, bicinechoninic acid

BES, N,N-bis(2-Hydroxyethyl)-2-aminoethanesulfonic acid

BICINE, N,N-bis(2-Hydroxyethyl)glycine

bp, base pair

C-terminus, carboxyl terminus

CD, circular dichroism

CHES, 2-(N-Cyclohexylamino)ethanesulfonic acid

CTP, cytidine-5'-triphosphate

dATP, 2'-deoxyadenosine-5'-triphosphate

dGTP, 2'-deoxyguanosine-5'-triphosphate

dCTP, 2'-deoxycytodine-5'-triphosphate

dTTP, 2'-deoxythymidine-5'-triphosphate

DEAE, diethylaminoethane

DMSO, dimethylsulfoxide

EDTA, ethylenediaminetetraacetic acid

EF_1, *Escherichia coli* F_1-ATPase

EGTA, ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid

F_1I, F_1-ATPase inhibitor protein

FTIR, Fourier transform infrared spectroscopy

g, gravity

GDP, guanosine-5'-diphosphate

GTP, guanosine-5'-triphosphate

hCA, human carbonic anhydrase isozyme II

hCA:ε, human carbonic anhydrase isozyme II - *E. coli* ε subunit fusion protein

IDP, inosine-5'-diphosphate

IPTG, isopropylβ-D-thiogalactoside

ITP, inosine-5'-triphosphate

kb, kilobase pair

K_{cat}, catalytic rate constant

k_d, dissociation constant

K_i, inhibition constant

K_m, Michaelis constant

M, molar

MES, 2-(N-morpholino)ethanesulfonic acid

M_r, relative molecular mass

N, normal

N-terminus, amino terminus

NADH, nicotinamide adenine dinucleotide

NMR, nuclear magnetic resonance

OSCP, oligomycin sensitivity conferring protein

PABA, *para*-aminobenzamidine

PAGE, polyacrylamide gel electrophoresis

PEP, phosphoenolpyruvate

P_i, inorganic phosphate

PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid)

pK_a, negative log of the acid dissociation constant

PMSF, phenylmethanesulfonylfluoride

PVDF, polyvinylidenedifluoride

SDS, sodium dodecylsulfate

TNP-ATP, 2'(3')-*O*-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate

TRICINE, N-tris(hydroxymethyl)methylglycine

Tris, tris(hydroxymethyl)amino methane

UTP, uridine-5'-triphosphate

V_{max}, maximum velocity

XTP, xanthosine-5'-triphosphate

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Directed amino acid substitutions were constructed for H211 of the *Saccharomyces cerevisiae* F_1-ATPase β subunit gene (*ATP2*). The mutations (H211N, H211D, H211I, H211K, and H211A) were expressed separately in *atp2::LEU2* hosts and the resulting F_0F_1-ATPases characterized. Yeast expressing mutation H211N showed respired aerobically; the other mutant strains did not. These phenotypes were suppressed intragenically by the mutation L203F, implying a physical or functional interaction between residues 203 and 211. This supports a three-dimensional model placing L203 and H211 in proximity. The mutant ATPases were unstable and could not be purified. Compared to wildtype, mutant ATPases were inhibited by ethanol and 2-propanol, showed reduced oligomycin sensitivity, had altered nucleotide specificities, elevated K_ms for ATP

hydrolysis, and low pH optima. Enzymes from mutant strains H211N, H211D, and H211K showed substantial decreases in the pK_a of the catalytic general base relative to wildtype. This work demonstrates that H211 and F203 are important for the stability of the wildtype enzyme complex. H211 is required for the proper assembly of F_1F_0, and also contributes to the structure of the active site although it is not required in the catalytic mechanism and its role is probably not dependent upon its ionization state.

In collaborative studies, recombinant *E. coli* F_1-ATPase ε subunit and recombinant beef heart F_1-ATPase inhibitor protein (F_1I) were evaluated. Recombinant F_1I inhibited beef heart F_1-ATPase noncompetitively (K_i = 0.2 μM) in a turnover- and pH-dependent fashion, and had no effect on *E. coli* F_1-ATPase. The amphiphilic α-helical region from R35-L45 of F_1I was targeted by alanine scanning mutagenesis. Substitutions in the proposed hydrophobic face of this helix interfered with inhibition by F_1I while mutations on the hydrophilic face had little effect. It was concluded that specific hydrophobic interactions are required for inhibition by F_1I. Recombinant ε inhibited *E. coli* F_1-ATPase noncompetitively (K_i = 11 nM) in a turnover- and pH-independent fashion which was also independent of the presence or absence of an N-terminal 30 kd fusion protein. It was concluded that the N-terminus of ε does not interact with F_1 and that F_1I and ε inhibit their respective ATPases through different mechanisms.

CHAPTER 1

INTRODUCTION

Plants and other photosynthetic organisms transduce electromagnetic energy into the chemical energy which is used in the biochemical processes of virtually all living things. This energy is transferred between organisms primarily as carbohydrates, fats, amino acids or proteins. Within cells chemical energy is stored in a variety of molecules. The processes of metabolism require the transfer of energy between these molecules. More of these transfers are mediated by the high energy compound adenosine-5'-triphosphate (ATP) than by any other molecule. ATP is one of many cellular compounds containing high energy phosphate bonds which serve as a source of free energy for the catalysis of reactions which would otherwise be thermodynamically unfavorable. The hydrolysis of ATP has an intermediate ΔG value relative to the other high energy phosphate compounds common in biological systems. This allows ATP and its hydrolysis products to donate and receive phosphate bond energy and thereby to act as an energy shuttle.

The F_0F_1 ATPase/synthase is a multisubunit transmembrane protein which couples the energy of an electrochemical gradient to the synthesis of ATP in the final step of oxidative phosphorylation. The F_0 portion of the complex spans the inner mitochondrial membrane and functions to conduct protons across it. This process

results in the collapse of the transmembrane electrochemical potential created by the electron transport chain. The subunits of F_1 and F_0 act together to couple the energy released by the transport process to the synthesis of ATP by F_1. The precise mechanism of energy transduction has not yet been determined and its elucidation is the ultimate goal in the study of oxidative phosphorylation. The enzyme is ubiquitous and is found in mitochondria, chloroplasts and bacteria. It has been estimated that the most frequently occurring enzyme catalyzed reaction on earth is the synthesis of ATP by F_OF_1 ATPases (Senior, 1988). Accordingly, the study of this enzyme constitutes a major field of biochemistry, and the volume of literature concerning this topic is substantial. A brief general review is provided by Boyer (1987), and more detailed general reviews by Senior (1988) and Kagawa (1984). Recent reviews focusing on relationship of structure and function and on the role of symmetry are also available (Cross, 1988, Ysern, Amzel, and Pedersen, 1988, and Tiedge and Schafer, 1989). For reviews dealing more closely with the enzyme mechanism see Futai et al., (1989), Boyer (1989), Penefsky and Cross (1991), and Hatefi and Matsuno-Yagi (1992). Extensive reviews dealing specifically with the *E. coli* enzyme have been written by Senior (1990) and Fillingame (1990). This brief introduction will summarize aspects of the structure, assembly, and catalytic mechanism of the F_0F_1-ATPases with an emphasis on F_1.

Structure

Capaldi and coworkers have used electron microscopy to describe the molecular architecture of the *E. coli* F_0F_1-ATPase (Gogol et al., 1987, Gogol et al., 1989a, Gogol et al., 1990, Aggeler et al., 1992a). The results of these authors are consistent with early reports depicting the gross structure of the mitochondrial enzyme (Soper et al., 1979, and Tzagoloff and Meagher, 1971). The F_1 moiety appears to be a 9 nm by 11 nm knob-like structure which is separated from the membrane spanning F_0 portion by a narrow stem 4.5 nm in length and 2 nm wide. F_1 can be stripped from F_0 and exist as a soluble complex capable of catalyzing the hydrolysis of ATP but not its synthesis. Soluble F_1 from rat liver mitochondria has been crystallized and X-ray mapped to a resolution of 3.6 angstroms (Bianchet et al., 1991). This complex is 7.6 nm "high" and 12 nm wide when viewed on a plane which would be parallel to the membrane, and 12 nm in diameter when viewed on a plane perpendicular to the membrane. The subunit structure of F_0F_1 is discussed below.

F_0 Subunits

In yeast (Devenish et al., 1992) and bovine (Hatefi and Mitsuno-Yagi, 1992) mitochondria, F_0 is made up of six and seven different subunits, respectively. *E. coli* F_0 contains only three different subunits, *a*, *b*, and *c* (Foster and Fillingame 1979). Subunits 6, 8, and 9 of yeast F_0 and subunits 6, *b*, and *c* of bovine F_0 appear to be equivalent to *E. coli a*, *b*, and *c*, respectively, and are thought to

form the transmembrane proton channel. The precise functions of the remaining yeast and bovine subunits are unknown, although it seems likely that some or all of them should be involved in forming the stalk which joins F_0 and F_1. In yeast, these subunits have been referred to as the F_A complex by Nagley (1988) and are considered to be separate from F_0 but still involved in the coupling of proton translocation to ATP synthesis.

The picture in *E. coli* is clearer and simpler. The transmembrane pore through which protons are conducted consists of one copy of subunit *a* having 5 to 8 transmembrane helices, and 9 to 12 copies of subunit *c*, each containing two transmembrane helices joined by a polar loop (Foster and Fillingame, 1982, Senior and Wise 1983, Cain and Simoni 1989, Walker et al., 1984, Lewis et al., 1990). There are also two copies of subunit *b* per complex. Each *b* subunit is predicted to have a single transmembrane helix and two long hydrophilic helices which project toward F_1 and may, together with subunit δ, form part of the stem structure (Porter et al., 1985). All three F_0 subunits are required for the assembly of a functional proton pore (Schneider and Altendorf, 1985).

The *a* subunit (M_r 30,285) has been extensively mutagenized and it appears that the C-terminal third of the protein is involved in proton conduction. In particular, three residues, R210, E219 and H245, (each protonatable) have been identified as crucial for this process (Cain and Simoni 1986, 1988, 1989). Single mutants *a*R210, *a*E219H and *a*H245E have 0%, 20% and 45% of wild type proton

translocation efficiency, respectively. Interestingly, the double mutant *a*E219H,H245E has about 50% of wild type proton translocation efficiency. This partial suppression of the translocation defect was interpreted as evidence of a functional interaction between residues 219 and 245, and also as evidence of physical proximity. Deletions of hydrophilic domains in the N-terminus of the *a* subunit show that the first 35 to 60 residues are important for membrane targeting and insertion (Lewis and Simoni, 1992).

Subunit *b* has been overexpressed and purified (Dunn, 1992). The relative molecular mass of this protein is 17,202. Chemical crosslinking studies combined with sedimentation equilibrium, size exclusion chromatography, and circular dichroism measurements suggest that the purified *b* subunit forms an α helical dimer in solution. The dimer is capable of inhibiting the binding of F_1 to naked F_0 indicating an interaction with soluble F_1. Proteolysis of F_1-depleted membrane vesicles results in the removal of the hydrophilic portion of subunit *b*. This does not affect F_0 proton translocation (Perlin, et al., 1983), but does eliminate F_1 binding in reconstitution experiments (Steffens et al., 1987), lending further support to the idea that the *b* subunit binds F_1.

Subunit *c* is a small protein (M_r 8,264) which, together with subunit *a*, is thought to compose the transmembrane proton pore. Modification of *c* with dicyclohexylcarbodiimide (DCCD) occurs at residue *c*D61 and results in a blockage of proton translocation (Hoppe and Sebald, 1984). This charged residue is predicted to be located in

the middle of one of the two transmembrane helices of *c*. Mutations of this residue to uncharged amino acids have also eliminated proton transduction (Hoppe et al., 1980, 1982). These data suggest that *c*D61 may be involved directly in the translocation of protons through F_0. It is interesting that DCCD modification of only one of the 9 to 12 *c* subunits present in F_0 is required to inactivate proton translocation (Hermolin and Fillingame, 1989). This is suggestive of a cooperative interaction between *c* subunits. If proton translocation involves the interaction of a single *a* subunit with each of the *c* subunits, then a proton relay model can be envisioned where the *c* subunits bind protons on the outer membrane side and transfer them into the center of the membrane spanning region. At this point the protons can be handed off to the *a* subunit and subsequently transferred through the membrane. This type of model would require the *a* subunit to be surrounded by *c* subunits and would also require either rotation of the *a* subunit within the *c* complex or rotation of the *c* complex around *a*.

F_1 Subunits

F_1-ATPases uniformly consist of nine subunits of five different types. The subunits are named in order of decreasing M_r, and are present in the complex in the ratio 3 α: 3 β: γ: δ: ε (TABLE 1.1). The F_1 inhibitor protein (F_1I) is loosely associated with F_1 and will copurify with it at neutral pH. This subunit will be discussed in a later section in the context of catalytic regulation.

Table 1.1

Precursors and Mature Subunits of the F_1-ATPase Complex.

	Yeast Mitochondria			Bovine Mitochondria			*E. coli*	
Subunit	P	M	M_r	P	M	M_r	M	M_r
α	580	544	58,500	553	510	55,164	509	55,200
β	511	492	54,575	528	480	51,595	459	50,155
γ	?	309	34,000	299	272	30,141	286	31,428
δ	?	121	11,000	168	146	15,065	177	19,328
ε	62	61	8,600	51	50	5,652	132	14,920
OSCP	212	195	20,870	213	190	20,968		
F_1I	?	63	7,383	109	84	9,572		

The length of precursor and mature subunits is given as the total number of amino acids. P: subunit precursor, M: mature subunit, M_r: relative molecular mass, OSCP: oligomycin sensitivity conferring protein, F_1I: mitochondrial F_1 ATPase inhibitor protein. E. coli δ is equivalent to mitochondrial OSCP. E. coli ε has sequences which are homologous to both mitochondrial δ and F_1I.

The results of chemical crosslinking (Satre et al.,1976) and electron micrographic studies (Tiedge et al., 1985) suggest that the α and β subunits alternate with each other in a hexagonal arrangement. This arrangement has been confirmed by the 3.6 angstrom crystal structure of rat liver F_1 (Bianchet et al., 1991) which shows two slightly offset layers of three subunits each, one layer composed of α subunits and one composed of β subunits. In each layer the subunits are arrayed about a 3-fold axis of symmetry, the center of each subunit separated from the others by 120°. The dimensions of the individual subunits are 4.8 X 4.8 X 5.0 nm for α and 4.0 X 4.8 X 5.0 nm for β. The two layers interdigitate so that the "height" of the complex is only 7.4 nm instead of 10 nm. Electron microscopic studies show a central cavity in the hexagon which is occupied by an off-center mass which is thought be composed of the γ subunit and either or both of the other minor subunits δ and ε (Tiedge et al., 1983, Boekema et al., 1986, Gogol et al., 1989b). The crystal structure of rat F_1 shows no central cavity. This may be an artifact of the imaging process, e.g. the image of the asymmetrically located mass is averaged out and consequently fills the central cavity. Heavy metal labeling of cysteine residues supports this interpretation. There are seven cysteine residues in rat F_1, two in each α subunit and one in γ, but there are nine heavy metal atoms detected in the X-ray map. Significantly, three of these atoms are symmetrically arrayed near the center of the complex where electron micrographs show the central mass to be. If the γ subunit is

asymmetrically positioned in the central cavity, and an average image of thousands of crystals is generated, then three heavy metal atoms would appear symmetrically arranged in the center of the hexagon. It should be noted that the authors considered this explanation of their data to be unlikely. They expected the minor subunits to appear as areas of low or discontinuous electron densities if at all, and they fail to detect such areas. The issues of whether or not the minor subunits compose the mass in the center of mitochondrial F_1, and which subunit owns the extra cysteines await resolution by further research.

Sequence homology between F_1 subunits from a variety of different organisms is relatively high (Walker et al., 1985) reflecting an evolutionary conservation of structure and function. This is not to say there are no substantial differences between enzymes from different organisms. For example, mitochondrial and *E. coli* enzymes differ in their F_0 sectors, as discussed above, as well as in F_1. The α, β and γ subunits of these enzymes are fairly homologous, and the *E. coli* δ subunit seems to correspond to mitochondrial oligomycin sensitivity conferral protein (OSCP). However, the *E. coli* ε subunit has sequence homology with both the mitochondrial δ subunit and the loosely associated mitochondrial inhibitor protein (F_1I), and there is no known *E. coli* equivalent to the mitochondrial ε subunit. Sequence similarity also exists within F_1 between the α and β subunits which appear to have evolved from a common ancestor (Walker et al., 1982, 1985).

F_1 α subunits from various species generally range between 55 kd and 58.5 kd and are present in three copies per F_1 complex. These subunits contain a consensus nucleotide binding site including a GX_4GKT/S motif, and have been shown to bind ATP very tightly with no requirement for Mg^{2+}(Cross and Nalin, 1982, and Issartel et al.,1986). The characteristics of the catalytic nucleotide binding sites include an affinity for a variety of nucleoside triphosphates, a requirement for a divalent cation, and free exchange with medium nucleotides. Because the α subunit binding site is specific for ATP, does not exchange nucleotides freely with the medium, and has no Mg^{2+} requirement, it cannot be a catalytic site. The actual role of the α subunit nucleotide binding site is unknown at this time, although a recent report suggests that binding of nucleotides to noncatalytic sites is required for catalytic positive cooperativity and is responsible for negative cooperativity of substrate binding (Jault and Allison, 1993). It is not yet clear whether this noncatalytic site is actually associated with the α subunit. It is also not certain that the nucleotide binding site attributed to the α subunit is contained entirely within that subunit. It has been suggested that it is actually a shared site, located at the interface of α and β subunits and composed of sequences from each (Kironde and Cross, 1987, and Wise et al., 1987).

The relationship of α subunit structural features to function has been reviewed by Senior (1990). The N-terminal portion of *E. coli* α has been implicated in binding the δ subunit (homologous to

mitochondrial OSCP) to F_1 (Dunn et al., 1980 , and Maggio et al., 1988). When the N-terminal 15 amino acids are removed from α, the F_1 complex does not bind δ and consequently cannot bind to F_0. On the basis of sequence homology to other nucleotide binding proteins (Walker 1982), mutational analyses (Rao et al., 1988b), and labeling by nucleotide analogues (Wise et al., 1987), the nucleotide binding site has been assigned to the region of residues 160-340. Senior (1990) has proposed that the segment of the α subunit from residues 345 to 375 is part of an interfacial surface with the β subunit, and that this region is involved in transmitting conformational changes between α and β subunits. Mutations in this region have been shown to diminish both positive catalytic cooperativity and negative cooperativity of substrate binding, while having no effect on unisite catalysis (Maggio et al., 1987). Jault et al., (1991) report a mutation at position E173 in the nucleotide binding region of the α subunit which also diminishes negative cooperativity. This mutation is far outside the so-called signal transmission region but may still be at an interface between α and β if the nucleotide binding site is shared. In this case it is conceivable that this residue could be involved in direct intersubunit communication. One other potential function of the α subunit is its possible role as a chaperone to the β subunit. This role is discussed below in the context of assembly.

The *E. coli* F_1 γ subunit has been shown by electron microscopy to occupy the center of the hexagonal array of α and β subunits

(Gogol et al., 1989b). The γ subunit is required for assembly of F_1 *in vivo* (Miki et al., 1988), and *in vitro* (Dunn and Futai, 1980) and has been crosslinked to both the α and β subunits (Aggeler and Capaldi, 1992) as well as to ε (Aggeler et al., 1992b). Purified F_1 subunits from either *E. coli* or thermophilic bacterium PS3 can be reassembled to form functional ATPases (Futai, 1977, and Yoshida et al., 1977). In *E. coli* F_1 the γ subunit is required for functional reconstitution, the minimal complex required for activity being $\alpha_3\beta_3\gamma$. PS3 subunits will form several functional oligomers including $\alpha_3\beta_3$, $\alpha_3\beta_3\gamma$, and $\alpha_3\beta_3\delta$ (Yokoyama et al., 1989). Of these, the $\alpha_3\beta_3\gamma$ complex has the greatest activity. The significance of these observations may simply be that the γ subunit is required for conformational stability of the α/β hexamer, or perhaps it is involved in the communication of conformational changes. The function of γ is not yet clear although it appears to be involved in energy coupling in the F_0F_1 complex. Shin et al. (1992) have isolated mutations at position 23 of the *E. coli* γ subunit (γM23R and γM23K) which disable proton pumping without affecting ATPase activity. Nakamoto et al. (1993) have generated several suppressor mutations of γM23R and γM23K by random mutagenesis of the entire *unc G* coding sequence. The suppressor mutations alter amino acids located in the C-terminus of γ primarily in the region from Q269 to V280. The authors argue that suppression implies a physical interaction between the regions surrounding the mutated residues. This serves as a basis for a model of a portion of the γ subunit. Secondary structure predictions suggest

that M23 and the loci Q269-V280 are located in α-helices. The authors claim that these helices are in proximity in the tertiary structure of γ, and that they both play a role in energy coupling.

The δ subunit of mitochondrial ATPases bears sequence similarity to the *E. coli* ε subunit and the two are considered to be equivalent (Walker et al., 1985). No function has been ascribed to the mitochondrial δ subunit yet, but crosslinking experiments show that it is located near the γ subunit in F_0F_1 (Joshi and Burrows, 1990). The *E. coli* ε subunit is required for binding of F_1 to F_0 (Sternweis, 1978) and may also regulate ATPase activity. *E. coli* ε is not bound to F_1 as tightly as the other subunits and will dissociate from the soluble enzyme with k_d in the nanomolar range. Mitochondrial δ is bound more tightly and is not observed to dissociate from the complex any more readily than the other subunits. Dunn et al. (1987) have utilized monoclonal antibodies directed against *E. coli* ε to completely deplete F_1 of the ε subunit. A comparison of K_{cat} values for ATPase activities of ε-depleted and ε-replete soluble F_1 shows that the association of ε with F_1 decreases K_{cat} by about 6-fold (Dunn et al., 1987). Soluble F_1 is inhibited by epsilon in a noncompetitive manner. Dunn et al. (1982), using ε-depleted F_1, have calculated the K_i for ε to be 0.3 nM. Previous studies using enzyme only partially depleted of ε reported k_i in the 10 nM range (Smith and Sternweis, 1977, and Sternweis and Smith, 1980), thus suggesting the possibility that ε has a regulatory role in the catalytic mechanism. *E. coli* mutants which express no ε subunit grow very

poorly and have lower concentrations of cellular ATP than wild type strains (Porter et al., 1983, Klionsky et al., 1984). On the other hand, Sternweis and Smith (1980) have shown that reassociation of F_1 with F_0 relieves the inhibitory effect of ε, suggesting that inhibition by ε may be artifactual. However, Mendel-Hartvig and Capaldi (1991) report that proteolysis of ε in F_1F_0 results in an increase in the rate of ATP hydrolysis. Klionsky et al. (1984) have suggested that the function of ε is to inhibit the ATPase activity of F_1 moeities not yet bound to F_0. While this may be one of the functions of ε, it seems unlikely that it would be its only function. Why would the cell evolve a protein which is a stable part of the assembled F_0F_1 complex, but whose only function is preventing premature ATP hydrolysis by unassembled F_1?. It would make more sense if it had a role in F_0F_1 as well. Mendel-Hartvig and Capaldi (1991) report that the conformation of the ε subunit changes when the enzyme binds nucleotides in the catalytic binding site. They combine this evidence with the fact that ε decreases the rate at which P_i dissociates from the enzyme (Dunn et al., 1987, and Wood et al., 1987), and propose that the primary function of ε is in coupling events at the catalytic sites with proton pumping.

The mitochondrial ε subunit is the smallest subunit of F_1 with M_r of 5652 in bovine heart and 8600 in yeast. Genes encoding the mitochondrial ε subunit from both bovine heart (Vinas et al., 1990) and yeast (Guelin et al., 1993) have been cloned and sequenced. The mature protein has been isolated from several sources including pig

heart (Penin et al., 1990, Gagliardi et al., 1991), beef heart (Walker et al., 1985), yeast (Arselin et al., 1991), and sweet potato (Kimura et al., 1989). The available primary structure information shows that these proteins are homologous. The ε N-terminus has the characteristics of both a mitochondrial targeting sequence and a leader sequence, although only the N-terminal methionine is cleaved to form the mature subunit (Vinas et al., 1990, Guelin et al, 1993). Joshi and Burrows (1990) have used homobifunctional crosslinking reagents to show that beef heart ε is located close to γ in the F_0F_1 complex. The ε subunit from pig heart has been purified by reversed-phase high pressure liquid chromatography as a tightly bound heterodimer with the δ subunit (Penin et al., 1990, Gagliardi et al., 1991). The purified ε and δ subunits will reassociate to form a dimer which is indistinguishable by criteria of chromatographic behavior, circular dichroism spectrum, and intrinsic fluorescence from the εδ dimer purified directly from F_1. Intrinsic fluorescence measurements indicate that the only tryptophan in ε, which is located in the N terminus, is involved in the interaction with δ.

Guelin et al. (1993) have constructed a yeast strain in which the chromosomal copy of the ε gene (*ATPε*) has been disrupted. The resulting strain is constitutively anaerobic and has no oligomycin-sensitive ATPase activity. The mutation effectively uncouples proton transport from ATP synthesis. The absence of ε causes a proton leak in F_0, possibly by influencing F_0 conformation. The resulting F_1 moiety is unstable and cannot be purified by extraction with

chloroform. The mutation is complemented by transformation of the mutant strain with an expression vector for *ATPε*. These results show that the ε subunit of mitochondrial ATPase is required for proper assembly and activity of the complex, in particular functional coupling of F_1 and F_0 requires the presence of ε.

The data presented in the previous three paragraphs show that the mitochondrial γ, δ and ε subunits are all closely associated by criteria of crosslinking or copurification. The *E. coli* γ subunit is homologous to the mitochondrial γ subunit and has been located in the center of the α/β hexagon. It therefore seems almost certain that the central mass detected in electron micrographs of F_1 is composed of one or more of the minor subunits.

The oligomycin sensitivity conferral protein (OSCP) is a member of the mitochondrial F_0F_1-ATPase complex and is located in the stalk region which connects F_0 to F_1 (McClellan and Tzagoloff, 1968). OSCP facilitates the binding of F_1 to F_0 in mitochondrial ATPases (Tzagoloff, 1970, and Penin et al., 1986) and is required for binding in the case of yeast (Uh et al., 1990). It has been shown to bind to F_0 (Dupuis and Vignais, 1987) and to F_1 independently, and has been crosslinked to the α and β subunits with zero-length crosslinkers (Dupuis et al., 1985). The *E. coli* δ subunit is homologous to OSCP and appears to have a similar function and location within the complex (Sternweis and Smith, 1977). F_0F_1 complexes which have been depleted of OSCP are incapable of ATP synthesis or P_i-ATP exchange, but this situation can be reversed by the addition of

exogenous OSCP. Genes encoding bovine (Joshi et al., 1992) and yeast OSCP (Uh et al., 1990) have recently been cloned. In the yeast system the clone was used to construct a chromosomal deletion strain which was incapable of aerobic respiration and which had no detectable oligomycin-sensitive ATPase activity. The bovine protein was expressed in E. coli, purified, and shown to have biological activity. A series of C-terminal deletion mutants was then generated by inserting stop codons 10, 20, 30, or 40 codons upstream from the authentic stop codon. Each of the resulting proteins was expressed and purified, and found to be ineffective in restoring ATPase or P_i-ATP exchange. Unfortunately the authors did not convincingly show that the mutant proteins were folded properly, so at this time there is no information concerning the function of specific regions of the protein.

F_1-ATPase β subunits are present in three copies per complex and contain one nucleotide binding site each. The characteristics of these sites include a preference for adenine nucleotides, but an affinity also for GTP, dATP, ITP, XTP, CTP, and UTP (Pullman et al., 1960, and Schnizer and Schuster unpublished), a requirement for a divalent cation (Pullman et al., 1960), and frequent exchange of bound nucleotides with medium nucleotides (Cross and Nalin,1982, and Issartel et al.,1986). These are properties which are characteristic of catalysis by F_1, and it is generally agreed that the β subunits contain the catalytic nucleotide binding sites. This idea is supported by the unusually high degree of sequence homology seen

between β subunits of various ATPases (Walker et al., 1982, 1985), and by the fact that the isolated β subunits of *Rhodospirillum rubrum* (Harris et al., 1985) and possibly *E. coli* (Al Shawi et al.,1990) can catalyze ATP hydrolysis whereas isolated α subunits cannot. The β subunit contains a GX_4GKT/S motif and other regions of homology to known nucleotide binding proteins (Walker et al., 1982). Extensive mutagenesis of the β subunits of *E.coli* F_1 has helped to identify specific amino acid residues and general regions of the protein which are important for catalysis and assembly (reviewed in Senior 1988, 1990, and Futai et al., 1989). Table 1.2 lists several *E. coli* β subunit mutations and their consequences.

The use of nucleotide analogues capable of labeling nearby groups has aided in identifying amino acids proximal to the nucleotide binding site (Cross et al., 1987, Garin et al., 1986, Hollemans et al., 1983, Bullough, and Allison, 1986). In addition, sequence homology with other nucleotide binding proteins of known structure such as adenylate kinase, *ras* p21, and EF-Tu has provided a guide for the study of nucleotide binding by F_1 β subunits. Walker et al., (1982) have defined two regions of homology, referred to as A and B, which are present in many nucleotide binding proteins. The A region encompasses the GX_4GKT/S motif and the B region includes an aspartate residue which may be involved in stabilizing the complex of the divalent cation with the nucleotide. Thomas et al., (1992) have deleted these regions from cDNAs encoding the C-terminal three fourths of the rat β subunit, expressed the resulting proteins in

Table 1.2

Mutations in the β subunit of *E. coli* F_1-ATPase.

MUTATION	DEFECT	REFERENCE
C137S	catalysis	Kironde et al. (1989)
" Y	catalysis	"
G142S	catalysis, positive cooperativity	Parsonage et al. (1987)
" D	catalysis	Kironde et al. (1989)
G146S	catalysis	Kironde et al. (1989)
G149S	suppresses S174F	Miki et al. (1990)
" I	assembly	Senior & Al Shawi (1992)
G149I, G154I	"	"
G150A, A151G, T156S	catalysis	Takeyama et al. (1990)
A151P	twice wild type activity	Takeyama et al. (1990)
A151P, V153S, T156G	severely inhibited catalysis	Takeyama et al. (1990)
" V	catalysis	"
G152D	catalysis	Lee et al. (1991)
" R	"	"
G154I	assembly	Senior & Al Shawi (1992)
K155A	catalysis	Omote et al. (1992)
" S	"	"
" T	"	"
" Q	severely inhibited catalysis	Senior & Al Shawi (1992)
" E	"	"
K155-G-T156	severely inhibited catalysis	Takeyama et al. (1990)
T156A	severely inhibited catalysis	Omote et al. (1992)
" C	"	"
" D	"	"
" S	activity 50% greater than wild type	"
E161Q	catalysis	Lee et al. (1991)
" R	"	"
S174F	Mg^{2+} catalysis, positive cooperativity, no effect on Ca^{2+} catalysis	Noumi et al. (1984), Parsonage et al. (1987)
E181Q	severely inhibited catalysis	Senior & Al Shawi (1992)
E185Q	assembly	Noumi et al. (1987)
" K	"	"
E192Q	catalysis	Parsonage et al. (1988)
G207D	catalysis	Kironde et al. (1989), Senior & Al Shawi (1992)
M209I	catalysis, positive cooperativity	Parsonage et al. (1987)

Table 1.2 Continued

MUTATION	DEFECT	REFERENCE
G214R	assembly	Parsonage et al. (1987)
D242N	severely inhibited catalysis	Senior & Al Shawi (1992)
R246C	catalysis, positive cooperativity	Parsonage et al. (1987)
G251D	catalysis	Lee et al. (1991)
T285D	catalysis	Noumi et al. (1988)
A295T	suppresses S174	Miki et al. (1990)
" P	"	"
Y297F	none	Wise (1990)
D301V	assembly	Lee et al. (1991)
D302V	assembly	Lee et al. (1991)
Y331S	catalysis	Wise (1990)
" C	"	"
" G	"	"
" F	50% wild type activity, tyrosine-OH not required for catalysis	Parsonage et al. (1987), Wise (1990), Weber et al. (1992)
" A	catalysis	Weber et al. (1992), Wise (1990)
" E	"	Weber et al. (1992)
" L	"	"
Y354F	none	Parsonage et al. (1987)
R398H	aurovertin resistant, no effect on catalysis	Lee et al. (1989)
R398W	"	Lee et al. (1991)
R398C	"	"
L400Q	suppresses S174	Miki et al. (1990)
P403S,G415D	assembly	Senior & Al Shawi (1992)

E. coli, purified them, and assayed their affinity for the fluorescent nucleotide analogue 2'(3')-*O*-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP). The affinities for TNP-ATP were compared to that of an overexpressed and purified wild type β subunit. They report that only the absence of the A region affects nucleotide binding. The A region deletion mutant showed a 30-fold decrease in affinity relative to wild type, while the B region deletion mutant showed no change in affinity. Both of these proteins and the wild type subunit were assumed to have folded correctly on the basis of circular dichroism spectra and resistance to *E. coli* proteases.

Duncan et al. (1986) have proposed a three-dimensional model for the nucleotide binding site of *E. coli* β which is based on the crystal structure of adenylate kinase. These authors suggest that the nucleotide binding site is contained within the protein segment from G142 to P332 and includes six β strands which alternate with five α helices in the primary structure. This model was presented as an hypothesis and will remain hypothetical until a high resolution crystal structure of the β subunit is available. However, there are data which support its validity. Table 1.2 lists many mutations in this region which have a negative effect on catalysis. Included in this domain is the GX_4GKT/S motif which forms a loop between the first β strand and first α helix. The lysine residue in this loop is thought to interact with the γ-phosphate of ATP in the cases of adenylate kinase (Pai et al., 1977) and p21 (Pai et al., 1989). An involvement in catalysis has not been demonstrated, but mutations

of this lysine (Reinstein et al.,1988, Tian et al., 1990) and of the corresponding one in ATPase β subunits (Table 1.2, K155) have severe negative effects on catalysis without apparent structural perturbation. Furthermore, the crystal structure of adenylate kinase (Pai et al., 1977) shows that the adenine-ribose moiety of ATP is in a hydrophobic pocket composed of five amino acid residues, three of which are clearly conserved in F_1 β subunits. Weber et al., (1992) used fluorescence spectroscopy to show that the environment surrounding the adenine moiety in the catalytic site of *E. coli* F_1 was hydrophobic. These data certainly do not confirm the model but they are consistent with it. Even though it remains unproven, the β subunit nucleotide binding site model is useful in the design of experiments and in the speculative interpretation of data.

Assembly

In the case of mitochondrial enzymes, each of the F_1 subunits is encoded by a nuclear gene and is expressed initially as a precursor (Maccecchini et al., 1979, Lewin et al., 1980, Runswick et al.,1990, Vinas et al., 1990). These precursors must be delivered to the mitochondria, transported across two membranes, proteolytically processed, and assembled into a functional enzyme. The N-termini of the known precursors have the characteristics of a membrane targeting and insertion sequence, including predicted amphipathic secondary structure with positively charged residues opposite hydrophobic ones.

The import pathway of each nuclearly encoded F_0F_1 subunit is not known in detail, but the β subunit has served as a model system for mitochondrial protein import and it is likely that certain aspects of its import pathway are similar to those of the other subunits. β subunit precursors are folded in the endoplasmic reticulum and transported to a mitochondrion at a contact site between the inner and outer membranes. Association with $Hsp70^{SSA}$ and uncharacterized cytosolic factors (Pfanner and Neupert, 1990, Hartl and Neupert, 1990) results in an ATP-dependent unfolding of the precursors which then interact with signal sequence receptors (eg., MOM19, Sollner et al., 1989). Each precursor is then transferred to a general insertion protein (eg., ISP42, Vestweber et al., 1989) and transported through the mitochondrial inner membrane by an unknown apparatus. This transport is dependent on the existence of an electrical potential across the inner membrane, the matrix having a negative charge relative to the cytosol (Pfanner and Neupert, 1986). Signal sequences are cleaved by a mitochondrial matrix processing peptidase (Hawlitschek et al., 1988) accompanied by a processing enhancing protein (Yang et al., 1988). Mature subunits are thought to be removed from the translocation apparatus by $hsp70^{SSC1}$ (Kang et al., 1990 Scherer et al., 1990) which is associated with the matrix face of the inner membrane. The subunits are then refolded in an ATP-dependent process involving $hsp60^{MIF4}$ (Ostermann et al., 1989). Subsequent assembly of β subunits into

macromolecular complexes also involves hsp60^{MIF4} (Cheng et al., 1989).

It has been proposed that the F_1 α subunit acts as a chaperonin for the β subunit (Luis et al., 1990, Avni et al., 1991). Yuan and Douglas (1992) suggest that the α subunit is not a molecular chaperone but rather an "assembly partner" which influences protein import without influencing folding. They show that the α subunit is necessary for efficient import of precursor proteins, particularly the β subunit. The other F_1 subunits are imported more efficiently in strains where the β subunit is imported normally than in those from which it is absent or imported poorly. The α subunit therefore indirectly influences the import of the γ, δ and ε subunits. These authors propose a model in which the α subunit aids in the discharge of β from hsp70^{SSC1} and that this release is coupled to the immediate assembly of β subunits into the complex. This is consistent with the finding of Burns and Lewin (1986) that the β subunit is assembled soon after import.

The issue of how the subunits of F_0 and F_1 assemble into a functional complex has not been settled. The assembly of *E coli* F_1F_0 has been proposed to occur by either sequential addition of subunits (Cox et al., 1981, Cox et al., 1987), or by association of all of the F_1 subunits into the ATPase complex, and subsequent binding to previously assembled and membrane-integrated F_0 (Sternweis and Smith, 1977). Evidence consistent with the second model has been reported in yeast where functional F_1 moieties are assembled, and

associate with the inner mitochondrial membranes of strains which do not synthesize F_0 subunits 6, 8, and 9 (Orian et al., 1984). Lewin and Norman (1983) have shown that imported F_1 subunits are assembled into new complexes and do not replace subunits in existing complexes. In the yeast system accessory proteins have been identified which are involved in the assembly of F_0 and F_1, but which are not directly associated with the complex. The *ATP10* gene product is required for the proper assembly of F_0 (Ackerman and Tzagoloff, 1990), and the *ATP11* and *ATP12* gene products are required for the assembly of F_1 (Ackerman et al., 1992, and Bowman et al., 1991). It is probable that other proteins involved in the assembly of F_1F_0 will be identified in the future, and the complete understanding of the assembly process awaits their discovery.

Catalytic Mechanism

As stated above, the ultimate goal of the study of oxidative phosphorylation is to determine the mechanism by which the energy of a transmembrane electrochemical gradient is transduced into phosphate bond energy in the form of ATP. The process of transferring protons across a membrane is facilitated by F_0, and the energy released in that process is somehow coupled to the catalytic site on an F_1 β subunit. Simplistically there are two ways for this to happen. One involves a direct coupling in which protons are passed through F_0 and F_1 to a catalytic site where they abstract oxygen from P_i, thereby facilitating a nucleophilic attack by the β-phosphate of ADP (Mitchell, 1974). The other way involves indirect coupling by

transmission of conformational changes. In this conformational coupling system the protons transferred through F_0 cause conformational changes in the F_0 subunits which are then transmitted to F_1 subunits and finally to the catalytic site(s). Transfer of protons into F_1 is not required because the conformational changes in the catalytic sites drive the reaction chemistry.

In the early 1970s it was noted that in the absence of any protonmotive force the ATPase enzyme "idled." That is, both the ATP synthesis and hydrolysis reactions occurred without product release. This was discovered by ^{18}O exchange experiments in which F_1 loaded with unlabeled ATP was incubated in ^{18}O labeled water. The ATP was labeled with ^{18}O without being released from the catalytic site (Boyer et al., 1973). It turned out that the equilibrium constant for the interconversion of ADP + P_i and ATP on the enzyme was nearly 1.0. This implied that energy input was not actually required to drive the reaction chemistry. Boyer then proposed that the energy-requiring steps in ATP synthesis or hydrolysis were actually the binding of substrates and the release of products, and that these processes depended on conformational changes in the catalytic sites (Boyer et al., 1973, Kayalar et al., 1977). He proposed an alternating site model of catalysis which came to be known as the three site binding change mechanism. Two site versions of the model have also been proposed wherein the third nucleotide binding site is regulatory and not catalytic (Berden et al., 1992, Bullough et al.,

1987). A recent report from Zhou and Boyer (1993) on the mechanism of photophosphorylation by chloroplast ATP synthase is also consistent with either two-site or three-site models.

The binding change mechanism is cyclical and the three-site version can be summarized as follows. Three catalytic sites exist in F_1, one on each β subunit. At a given time one of these sites has tightly bound ATP, the second has tightly bound ADP and P_i in equilibrium with ATP, and the third is empty and in a conformation which will not bind substrates. Energy from the electrochemical gradient is then released and causes a conformational change in F_0 subunits which is transmitted to F_1. Each of the binding sites then undergoes a conformational change. The first site releases ATP and adopts the empty site configuration. The second site binds ATP tightly, and the third site adopts a conformation allowing it to bind ADP and P_i tightly. The cycle can then repeat. An important aspect of this mechanism is that it accommodates negative cooperativity of substrate binding and positive catalytic cooperativity, both of which are documented characteristics of catalysis by F_0F_1 (Grubmeyer and Penefsky, 1981, Cross et al., 1982).

Another important implication of the mechanism is the functional asymmetry of nucleotide binding sites. At no time do any two sites have the same characteristics, and each site must pass sequentially through each of the three possible conformational states. This functional asymmetry is paralleled by the structural asymmetry seen in electron micrographs (discussed above) in which a central

mass is associated with one α/β pair. Cryoelectron microscopy studies using monoclonal antibodies to *E. coli* ε and α subunits have shown that when ATP is bound in the absence of Mg^{2+} (a noncatalytic condition), the ε subunit associates with a β subunit independently of the location of the central mass (Gogol et al., 1990). However, in the presence of Mg^{2+} and ATP (under the conditions of catalysis), the central mass and ε subunit are usually associated with the same β subunit. These observations are consistent with the idea that the minor subunits are involved in the communication of conformational changes involved in catalysis.

The alternating positions occupied by the α and β subunits in the F_1 complex suggest that the α subunits are also involved in intersubunit communication, and therefore may play a role in the sequential changes in the conformation of each binding site. This idea is supported by mutational analyses of the α subunit. Senior's laboratory has isolated α subunit mutants which inhibit multisite catalysis (positive cooperativity) as well as negative cooperativity of substrate binding without affecting the total number of nucleotides bound (Wise et al., 1984). It seems clear that the catalytic mechanism of F_1-ATPase is extremely complex and, even though catalysis occurs on the β subunits, the kinetic characteristics depend on the interactions of all of the F_1 subunits.

Regulation

The mitochondrial ATP synthesis reaction is reversible *in vivo*. In constitutive anaerobes the ATPase functions as a proton pump.

The enzyme uses ATP to generate a membrane potential which is ultimately used for nutrient uptake. Mitochondria also run the hydrolysis reaction in order to reduce oxidized electron carriers. This information raises the issue of regulation. Biological systems need a mechanism for regulating the activity of the F_0F_1-ATPase in times where there is a need for a large cellular pool of ATP. If such a pool is accumulated at the expense of the proton gradient, and the concentration of ATP is far greater than that of ADP, then there needs to be a mechanism of avoiding a futile cycle. In the case of mitochondrial ATPases this regulation is achieved through the action of an F_1-ATPase inhibitor protein (F_1I). Inhibitor proteins have been discovered in a variety of sources including beef heart (Pullman and Monroy, 1963), rat liver (Cintron and Pedersen, 1979) *Saccharomyces cerevisiae* (Hashimoto et al., 1981), and *Candida utilis* (Klein et al., 1977). Proteins which may have a similar function include the ε subunits of *E. coli* (Smith and Sternweis, 1977) and chloroplasts (Nelson et al., 1972, and Nieuwenhuis and et al., 1974).

The interaction of F_1I with F_1 has been best characterized in the beef heart system. Bovine F_1I has been shown to bind to the β subunit of F_1 in a stoichiometry of one per F_1 complex (Klein et al., 1980, 1981, Wong et al., 1982), so it is likely that inhibition of ATP hydrolysis is mediated through a single β subunit. Current models of the interaction of the F_1I with F_1-ATPase depend on conformational changes in the inhibitor protein itself (Panchenko and Vinogradov, 1985, Fujii et al., 1983, Milgrom, 1991), and possibly the ATPase

complex (Schwerzman and Pedersen, 1986). The inhibitor protein can exist in at least two conformations, active and inactive. The active conformation predominates at acidic pH, and the inactive one at basic pH. Protonation of histidine residues has been implicated in the conformational transition of both bovine and yeast inhibitor proteins (Panchenko and Vinogradov, 1985, Fujii et al., 1983). The active form of the inhibitor inactivates F_1 immediately, whereas inhibition by the inactive form takes several minutes and is dependent on catalytic turnover (Schwerzman and Pedersen, 1986, Panchenko and Vinogradov, 1985). Conversion between active and inactive forms depends on pH *in vitro*, and is thought to depend on the state of mitochondrial energization *in vivo* (Van de Stadt et al., 1973, Harris et al., 1979, Husain and Harris, 1983). The inhibitor assumes its active form in mitochondria under nonenergizing conditions and reverts to its inactive form upon formation of the proton gradient. Milgrom (1991) has shown that F_1 inhibited by F_1I contains a single nucleotide trapped in a catalytic site. According to the binding change model, once one catalytic site is emptied by release of products an alternate catalytic site assumes the conformation required for product release (Kayalar et al., 1977). Milgrom (1989, 1991) suggests that the inhibitor protein functions by blocking product release at a single catalytic site, and that this eliminates the cooperative interactions necessary for promoted catalysis. This idea is supported by experiments showing that the

F_1I-F_1 complex is capable of unisite (noncooperative) ATP hydrolysis (Vasquez-Laslop and Dreyfus, 1990).

The picture that has been presented in this introduction is one of an extremely complex and important multisubunit enzyme. The work in the chapters that follow concentrates primarily on the role of a single amino acid in the β subunit of the yeast F_0F_1-ATPase. The data presented will show that this amino acid plays a role in maintaining the correct conformation of the β subunit and that it is essential for normal assembly and normal catalysis. Further data will provide support for an adenylate kinase-like configuration of the nucleotide binding site. The last chapter of this work focuses on the regulation of the beef heart F_1-ATPase. A collaborative study was undertaken to identify important amino acid residues in the beef heart F_1 inhibitor protein as well as to compare some of its characteristics to those of the inhibitory ε subunit of *E. coli.*

CHAPTER 2

PHENOTYPIC CHARACTERIZATION OF *atp2* YEASTS AND ANALYSES OF ASSEMBLY AND STABILITY OF MUTANT ATPASES

Introduction

The F_0F_1 ATPase/Synthases catalyze the formation of ATP in the final step of oxidative phosphorylation. The F_1 portion of the enzyme contains the catalytic domains and consists of five different subunits in an $\alpha_3:\beta_3:\gamma:\delta:\varepsilon$ stoichiometry. The catalytic nucleotide binding sites are found on the β subunits (Cross and Nalin, 1982). The structural constituients of these sites have not yet been rigorously identified due to the technical difficulty in obtaining high resolution NMR or X-ray crystallographic information for such a complex enzyme. However, analysis of the β subunit primary structure reveals a consensus sequence found in many other nucleotide binding proteins (Fry et al., 1986, Walker et al., 1985) (Fig.2.1). This sequence has been shown to form part of the nucleotide binding site in the enzymes adenylate kinase and *ras* p21. High resolution tertiary structures have been solved for both of these enzymes in the nucleotide-bound state (Fry et al., 1985, Fry et al., 1988, De Vos et al., 1988, Pai et al., 1989) and the consensus sequence has been divided into two contiguous regions, a glycine-rich loop followed by an α helix. The glycine-rich loop interacts with the polyphosphate chain of the nucleotide, and the α helix appears to

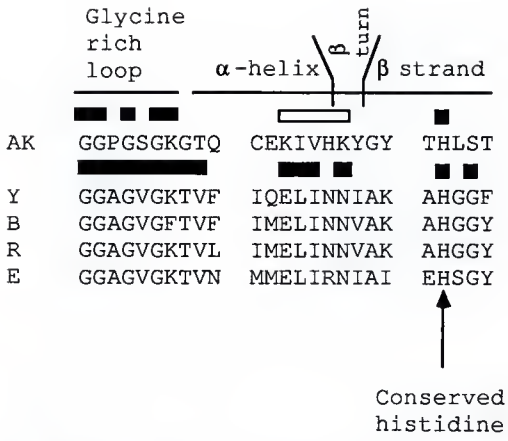

Fig. 2.1 Consensus sequence forming part of a nucleotide binding site. Sequences are compared from four sources, AK: adenylate kinase (Fry et al., 1988), Y: yeast F_1-ATPase β subunit (Takeda et al. 1985), B: bovine heart F_1-ATPase β subunit (Walker et al., 1982), R: rat liver F_1-ATPase β subunit (Garboczi et al., 1988), E: *E. coli* F_1-ATPase β subunit (Walker et al., 1982). Filled boxes represented absolutely conserved sequences. The open box represents a conserved motif consisting of one hydrophilic residue followed by two hydrophobic residues and another two hydrophilic residues. The conserved histidine corresponds to H36 of adenylate kinase, H211 of the yeast F_1-ATPase β subunit, H179 of the bovine β subunit, H177 of the rat liver β subunit, and H170 of the *E. coli* β subunit.

interact with the purine moiety of the nucleotide. The effects of mutations in the consensus sequence have been studied in several proteins including p21, adenylate kinase, and yeast and *E. coli* F_1-ATPase β subunits (Adari et al., 1988, Reinstein et al., 1988, Mueller, 1989a, 1989b, others in Table 1.2) Amino acid substitutions in this region generally lead to a decrease in catalytic activity.

Duncan et al. (1986) have proposed a three dimensional model for the nucleotide binding site of the F_1-ATPase β subunit which is based on secondary structure predictions from the β subunit amino acid sequence. The pattern of α helices and β strands in the region of the predicted nucleotide binding site is similar to that of adenylate kinase. The authors have therefore used the high resolution structures of adenylate kinase as a scaffolding for their model. Both X-ray crystallography and NMR spectroscopy have been employed in the structural mapping of adenylate kinase. These approaches have yielded virtually identical three dimensional structures of the native enzyme, but there has been controversy over the positioning of ATP in its binding site (Pai et al., 1977, Fry et al., 1985, Tian et. al, 1988, Tsai and Yan, 1991). One specific source of dispute has been the role of a particular histidine residue (H36) which happens to be conserved in F_1 β subunits. Early physical studies led to the conclusion that H36 was involved in the catalytic mechanism (discussed in Tian et al., 1988). The crystal structure of Pai et al. (1977) seemed to corroborate this conclusion, but the NMR studies of Mildvan and coworkers contradicted it (Fry et al., 1985,

1987). The NMR model predicts that the adenine moiety of MgATP interacts with a hydrophobic cleft composed of residues I28, V29, H36, L37, and L91. Residues I28 and V29 are located near the C-terminus of the α helix of the consensus sequence. A β turn occurs immediately after the end of this helix and a β strand follows (Figs. 2.2 and 2.3). H36 and L37 are located within the β strand and come into proximity with the helix. In this model H36 is not situated close enough to the polyphosphate chain to be involved in catalysis. The F_1 model of Duncan et al. adopts this binding configuration for ATP (Fig 2.3).

Tian et al. (1988) performed site-directed mutagenesis of adenylate kinase at position 36 and evaluated the kinetic and structural properties of the resulting enzymes. They found that the changes in kinetic constants caused by the mutations were insufficient to justify a role for H36 in the catalytic mechanism, but hydrophobic interactions between H36 and the adenine group of MgATP were not ruled out. The most striking characteristic of the mutant enzymes was their instability during purification. As the substitutions for H36 became less conservative the resulting proteins became more unstable, the order of stability being: H36>H36Q>H36N>H36G. The resistance of the purified enzymes to unfolding in the presence of guanidine hydrochloride was measured, and the same hierarchy of stability was observed. The authors concluded that while there may be hydrophobic interactions between

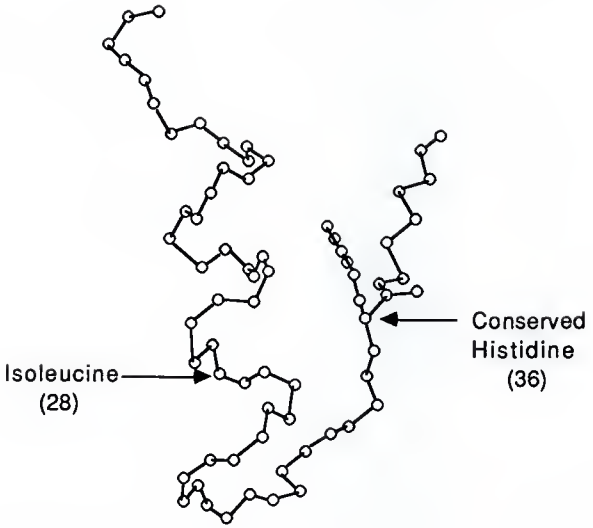

Fig. 2.2 The hydrophobic cleft of adenylate kinase. Redrawn from a high resolution structure presented in Fry et al. (1987). The structure shows the main chain atoms from G19 to T39. The imidazole ring of H36 is included and is shown projecting into the cleft. The α–carbons of I28 and H36 are shown. I28 and H36 are homologous to L203 and H211 of the yeast F_1-ATPase β subunit.

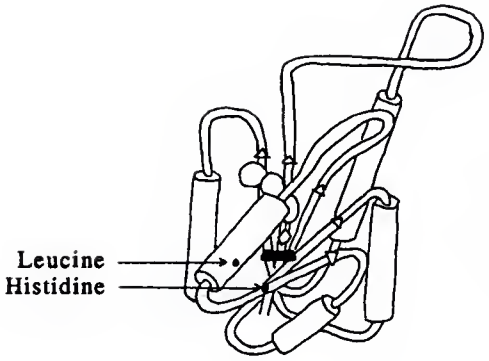

Fig. 2.3 A model of the *E. coli* F_1-ATPase β subunit nucleotide binding site. This model is redrawn from Duncan et al. (1986). The positions of amino acids homolgous to yeast β subunit L203 and H211 are shown, as is the predicted orientation of ATP in the binding site. Cylinders represent α helices and ribbons represent β strands.

H36 and the adenine moiety of MgATP, the most important role of H36 is in the structural stabilization of the enzyme.

Garbozci et al. (1988) have approached the study of the F_1 β subunit nucleotide binding site through the use of a synthetic peptide. These authors synthesized a 50 amino acid peptide homologous to the rat liver mitochondrial ATPase β subunit and studied its interaction with ATP. The sequence of the peptide encompassed both the glycine-rich loop and the α helical region of the proposed nucleotide binding site. When the peptide was added to a buffered solution containing 1 mM ATP, a precipitate formed which was composed of the peptide and ATP. Examination of the sequence of the peptide led to the suggestion that a histidine residue is involved in substrate binding by the interaction of its positive charge with a negative charge on the nucleoside triphosphate chain. The histidine in question is homologous to H36 of adenylate kinase. This idea is inconsistent with the NMR model of adenylate kinase and the kinetic results of Tian et al., but is consistent with the X-ray model. Fry et al. (1988) have examined the structure of a 45 amino acid peptide of adenylate kinase by 2-D NMR, FTIR, and circular dichroism spectroscopy. This peptide binds ATP and is homologous to the rat liver peptide described above. They find that the tertiary structure of this peptide is essentially the same as the corresponding segment of native adenylate kinase. Chuang et al. (1992) have performed similar studies with the rat liver F_1 β subunit peptide. They find that it has transient tertiary structure and does not

resemble the adenylate kinase peptide. These results suggest that the tertiary structure of this segment of the F_1 β subunit may be unlike the corresponding segment of adenylate kinase. If that is the case, then the conserved histidine may play a different role in F_1-ATPase than it does in adenylate kinase.

In the rat liver F_1-ATPase β subunit H177 is homologous to adenylate kinase H36. Thomas et al. (1992) have recently mutated rat liver βH177 to asparagine. A three-quarter length C-terminal β subunit fragment containing this mutation was expressed in *E. coli* and purified. This peptide binds the the fluorescent ATP analogue TNP-ATP with the same affinity and stoichiometry that the purified wild type full-length β subunit does. The H177N peptide was assumed to have folded correctly based on its solubility in *E. coli* and its resistance to *E. coli* proteases. Better evidence of correct folding would strengthen the conclusions drawn from this data, but the nucleotide binding results are convincing nonetheless. These data strongly suggest that H177 is not involved in substrate binding.

The resolution of the role of the conserved histidine in F_1-ATPase β subunits is the subject of chapters 2 and 3 of this thesis. This work requires a system in which this residue can be examined in the context of the F_0F_1 complex. In this respect, the peptide approaches of Garboczi et al. and Thomas et al. provide interesting preliminary data, but are not sufficient for firm conclusions concerning the role of this residue in the native enzyme. The yeast *Saccharomyces cerevisiae* provides a system which greatly facilitates

the study of single residues within the entire F_0F_1-ATPase by site-directed mutagenesis. The gene encoding the β subunit (*ATP2*) has been cloned and sequenced (Saltzgaber-Muller et al., 1983, Takeda et al., 1985), and yeast hosts which express no β subunit are available (Takeda et al., 1985). The well known variety of yeast shuttle vectors and selectable genetic markers allows flexibility in the design of experiments. For example, many recombinant genes are expressed in yeast from multicopy plasmids. In cases where gene dosage is important, the use of a single copy vector containing a fragment of a yeast chromosome is an option. Another advantage of the yeast system is their capacity for anaerobic growth. This allows the recovery of mutations which would be lethal in a constitutively aerobic organism. Yeast and other fungi also provide the model systems for studying the assembly of the mitochondrial F_0F_1-ATPase. The kinetic characteristics of the yeast enzyme are well established and the enzyme is easily prepared in large quantities in either membrane bound or soluble form (Takeshige et al, 1976). The yeast *Saccharomyces cerevisiae* has therefore been selected as the system in which to study the role of the conserved histidine of the F_1 β subunit in this work.

In the yeast F_1-ATPase β subunit the conserved histidine residue corresponds to H211. In this chapter, the role of H211 has been investigated by site-specific mutagenesis and subsequent analyses of the mitochondrial ATPases from the mutant yeasts. H211 has been replaced with an acid (aspartate), a base (lysine), a

polar structural analog (asparagine), a hydrophobic residue (isoleucine), and a residue lacking sidechain bulk (alanine). The rationale for these substitutions is as follows. If a positive charge is important to the function of position 211, then the substitution of aspartic acid for histidine should be extremely deleterious. On the other hand, the insertion of lysine would provide a positive charge at higher pH values than histidine does. This might result in a broadening of the catalytic pH optimum, or in a shift of this optimum to a higher pH value. The substitution of asparagine should provide an amide nitrogen in roughly the same spatial location as the nitrogen at position number 1 of the histidine imidazole ring. This substitution tests the role imidazole nitrogen 1. An alternative role of the histidine might be to provide hydrophobicity, perhaps to interact with the adenine moiety of ATP. If this is the case, then the substitution of isoleucine might impair function the least. The substitution of alanine for histidine merely tests the need for a large side chain at position 211 of the β subunit.

In addition to the mutations at position 211, a single mutation was engineered at position 203. This codon, normally encoding leucine, was altered to phenylalanine. L203 of the yeast F_1 β subunit is equivalent to I28 of adenylate kinase (Fry et al.,1986). This residue is predicted to form part of the hydrophobic cleft thought to interact with the adenine moiety of MgATP. In the high resolution structures of adenylate kinase it is located on the opposite side of the cleft from the conserved histidine (Figs. 2.2 and 2.3). This mutation

results in an increase in hydrophobic bulk and tests the size restriction on the hydrophobic group at position 203.

Briefly, the experimental approach was as follows. Oligonucleotide-directed site-specific mutagenesis was performed on *ATP2* to generate the mutations described above. The resulting genes were then expressed under the control of their natural promoter in the yeast host strain AVY4-1 The chromosomal copy of *ATP2* carried in AVY4-1 has been destroyed by insertion of a copy of *LEU2*, and it expresses no β subunit of its own. The phenotypes of the yeasts expressing the mutant β subunits were then characterized and the assembly and stability of the mutant ATPases was assessed.

The results of this chapter show that H211 is critical for maintenance of the conformation of the enzyme complex and that it is not required for catalysis. Furthermore, it is suggested that positions 203 and 211 have a functional, and possibly a structural, interaction. This supports the nucleotide binding site model of Duncan et al. (1986) which predicts that these residues are in proximity to each other.

Materials

Strains

E. coli NM522 (*sup*E, *thi*,Δ(*lac-pro*AB), Δ *hsd*59r-,m-),{F', *pro*AB, *lac*I$_q$ZΔM15}), and SCS-1 (F-, *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_k-, m_k+), *sup*E44, *rel*A1) were used to recover and propagate recombinant or mutated plasmids. L-broth (LB) consisted of 0.1% tryptone, 0.05% yeast extract, and 0.05% NaCl, and was supplemented

with 100 mg/l ampicillin for the selection of bacteria carrying plasmids of interest.

Yeast strains SEY2102 and AVY4-1 were gifts from Michael Douglas. SEY2102 was the source of wild type F_1-ATPase in this study, its genotype is *MAT* α ,*ura* 3-52, *leu* 2-3, 112, *his* 4-519, *gal* 2, *suc*2-sΔ9. AVY4-1 is derived from SEY2102 by insertion of *LEU2* into *ATP2* (*atp2* :: *LEU2*). This strain served as the host for expression of wild type and mutated forms of of the β subunit. Strains carrying mutated versions of *ATP2* are named for the particular mutation, for example the strain carrying the mutation H211N is simply named βH211N. AVY4-1 carrying wild type *ATP2* on pβOK (see below) is referred to as strain βOK. The wild type strain SEY2102 was transformed with a plasmid carrying *URA3* (pVTU103) to allow growth under the same conditions as the mutant strains. For simplicity this transformed strain was named SEY103. Yeast minimal media contained 0.67% Difco yeast nitrogen base, 20 mg/L adenine, complete amino acid supplementation, and 2% glucose. Ethanol and glycerol at 2% each were substituted for glucose when assaying aerobic respiration. Yeast were transformed with plasmids by the alkaline cation method (Ito et al., 1983) or by spheroplasting (Hinnen et al., 1978). Transformants were selected by uracil prototrophy.

Plasmids

Plasmid pTZ19R (Pharmacia) was modified as follows (Fig. 2.4.). A 195 bp BglI-SphI fragment was removed, the resulting ends were made blunt by treatment with T4 DNA polymerase and the

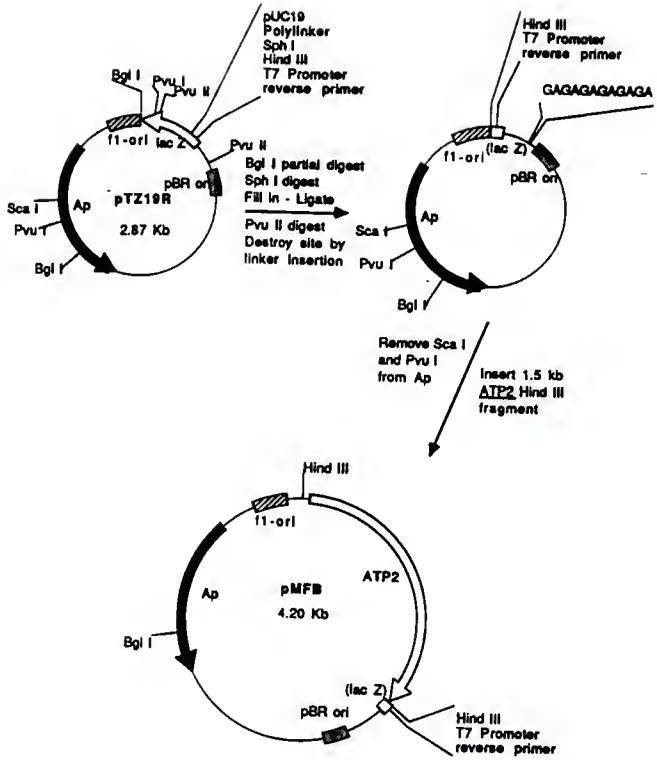

Fig. 2.4 Construction of pMFβ. Details of the procedure are given in Materials.

plasmid was reclosed. The PvuI and ScaI sites in the β-lactamase gene (Ap) were simultaneously removed by site directed mutagenesis as described below. The PvuII site at position 418 was destroyed by the insertion of a 12 bp double stranded oligonucleotide. The resulting 2.7 kb plasmid contains an f1 origin, Ap (ampicillin resistance), a T7 promoter, a reverse sequencing primer, a pBR322 replication origin, and no restriction sites which are unique in the sequence of *ATP2*. *ATP2* was inserted into this plasmid as a 1.5 kb HindIII fragment, and the resulting 4.2 kb plasmid, pMFβ (Fig 2.4), was used as the substrate for site-specific mutagenesis.

Plasmid pβOK-H was a gift from Michael Douglas. It is a multi-copy yeast shuttle vector containing a 2.8 kb yeast genomic Eco RI-HindIII fragment which carries *ATP2* and about 1.3 kb of 5' flanking sequence (Fig 2.5). All of the mutations constructed in this study were subcloned into this vector on restriction fragments which replaced the corresponding wild type restriction fragment. pVTU103 is a yeast shuttle vector containing *URA3* (Vernet et al.,1987). SEY2102 was transformed with this plasmid to allow growth on minimal media lacking uracil. pβOK-H, its derivatives, and pVTU103 were maintained in yeast by selection for uracil prototrophy.

Methods

Oligonucleotide-directed Site-specific Mutagenesis

Site-specific mutants were constructed by a gapped heteroduplex technique (Fig 2.6) (Morinaga et al., 1989). Mutations

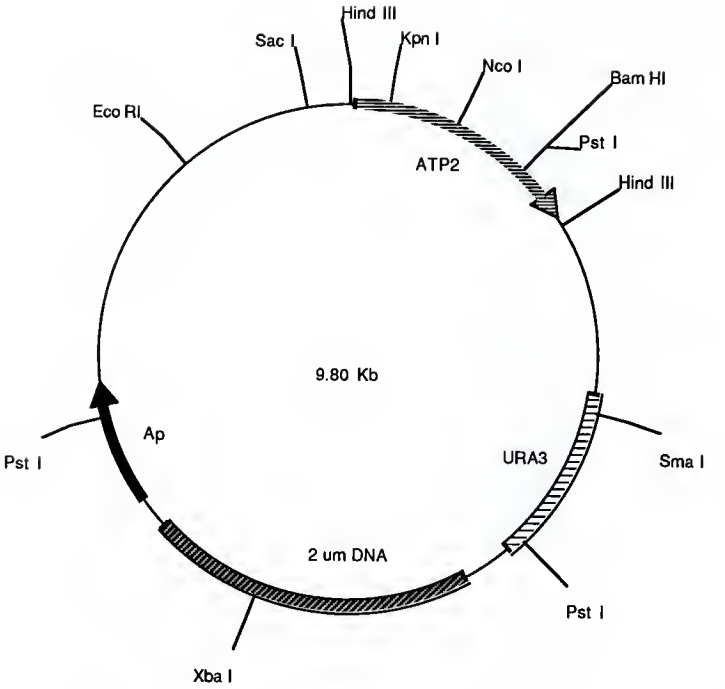

Fig. 2.5 Plasmid pβOK. The details of this plasmid are discussed in Materials.

were designed so that they always either removed or introduced a restriction site, this allowed enrichment for the desired mutation. For example, pMFβ was digested with restriction enzymes at sites on either side of the region to be mutagenized so that a fragment of about 500 bp or less was eliminated. The larger restriction fragment was then separated by agarose gel electrophoresis and eluted from the gel. Another sample of pMFβ was digested with a single restriction enzyme at a unique site outside of the 500 bp gap region. This sample was treated with calf intestinal phosphatase and purified by electrophoresis as above. Approximately 3 pmole of the two DNA fragments were then placed together in a microfuge tube with 50 pmole of mutagenic oligonucleotide. The solution was brought to 10 µl by the addition of 2 µl of a 10X ligase buffer (1 M NaCl, 65 mM Tris pH 7.5, 80 mM $MgCl_2$ and 10 mM β-mercaptoethanol) and the DNA was denatured by incubation for 3 minutes at 100° C. Single strands were allowed to reanneal and form gapped heteroduplexes during 30 minute incubations at 30° C and then 4° C. The solution was then incubated at 0°C for 10 minutes. The reaction volume was brought to 20 µl by the addition of dATP, dGTP, dCTP, and dTTP to 2.5 mM, ATP to 3.5 mM, beta mercaptoethanol to 8 mM, and 5 units each of Klenow fragment of DNA polymerase 1 and T4 DNA ligase. The reaction mixture was incubated at 11-16° C for 8 hours to allow polymerization and ligation of the gapped region. The resulting plasmids were used to transform *E. coli* NM522. Competent cells were prepared by the

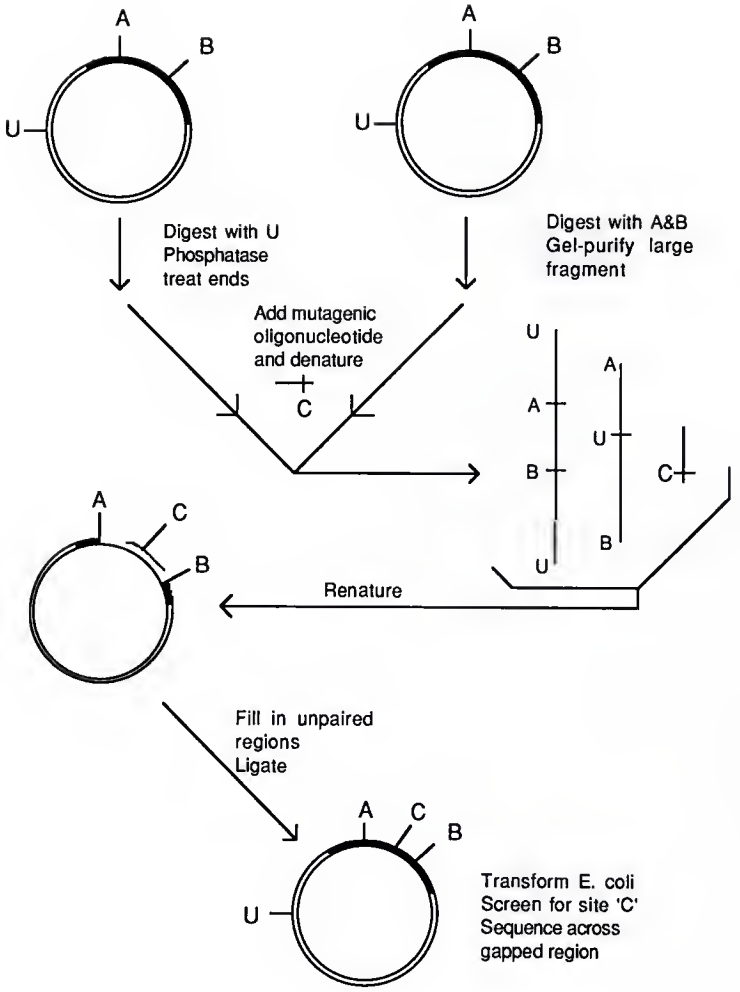

Fig. 2.6 The gapped heteroduplex technique of site-directed mutagenesis

method of Hanahan (1983). Usually about 200 colonies were picked and used to inoculate 20 0.5 ml cultures of L-broth with 100 mg/L ampicillin. Plasmid DNA was isolated from these heterogenous cultures by boiling lysis (Maniatis et al., 1982) and enriched for the presence or absence of a restriction site as follows. If a mutation resulted in the introduction of a restriction site, the plasmid was digested with an enzyme recognizing that site, separated from uncut plasmid DNA on an agarose gel and purified. The DNA was then ligated and passed through *E. coli* once again after which plasmids from individual colonies were screened for the introduction of the restriction site. DNA was isolated from positive clones by alkaline lysis, and the region which had constituted the gap in the heteroduplex was sequenced and then substituted as a cassette for the corresponding region of a wild type clone carried on pβOK-H.

If the desired mutation resulted in the removal of a restriction site, then the plasmid DNA from heterogenous culture was digested with the enzyme which recognized the site targeted for removal, and another enzyme for which the plasmid had a unique site. The resulting molecules were separated by electrophoresis and once cut plasmids were gel-purified, ligated, and used to transform *E. coli*. Single colonies were then screened for the absence of the site in question, those clones lacking it were sequenced, and positive clones were inserted into pβOK-H as described above.

Nucleotide changes in codon 211 were as follows: CAT (H211) was altered to AAT (H211N), GAT (H211D), AAG (H211K), ATT

(H211I), and GCT (H211A). These changes resulted in the elimination of a unique Nco I site. The nucleotide change in codon 203 was TTG (L203) to TTC (L203F). This resulted in the introduction of an Eco RI site. Mutagenic oligonucleotides and sequencing primers were synthesized and sequencing was performed by the core facilities of the University of Florida Interdisciplinary Center for Biotechnology Research.

Preparation of Submitochondrial Particles and Purification of ATPase

Yeast were grown in minimal media to an absorbance of 1.8 at 610 nm, collected by centrifugation at 3,000 X g for 5 minutes at 4° C, and resuspended in chilled breaking buffer (0.6 M sucrose, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM PMSF, 1 mM para-aminobenzamidine). All subsequent steps were carried out at 0-4° C. Yeast were broken in a Bead Beater (Bio-Spec products, Bartlesville, OK) using five breaking cycles of two minutes separated by 6-10 minutes cooling in ice water. Cellular debris was removed by centrifugation 2-4 times at 3000 X g and crude mitochondria were collected by centrifugation for 20 minutes at 20000 X g. The pellet was resuspended in breaking buffer at 5-20 mg protein/ml buffer, and submitochondrial particles were generated by sonic oscillation for 1 minute in a Heat Systems W-225 sonicator. Debris was removed by centrifugation at 20000 X g for 10 minutes. Submitochondrial particles were collected by centrifugation at 100,000 X g for 90 minutes, resuspended in breaking buffer at 5 mg protein/ml, and stored at -75° C.

F_1-ATPase was purified by chloroform extraction and DEAE anion exchange chromotography essentially as described (Mueller, 1988) except that gel filtration was omitted. Submitochondrial particles (10-20 mg protein/ml) were extracted with 0.5 volume of chloroform. ATP was added to 2 mM and debris was removed by centrifugation for 10 minutes at 2000 X g at room temperature. The aqueous phase was transferred to a 50 ml Corex tube and centrifuged at 48000 X g for 30 minutes at room temperature. The pH of the supernatant was adjusted to 7.5 with 0.1 N NaOH and was loaded onto a 10 X 2.5 cm column of Whatman DE-52 anion exchange resin which had been equilibrated with breaking buffer plus 2 mM ATP (buffer B). The column was washed with 100 ml of buffer B, and then washed with 100 ml of buffer B plus 20 mM K_2SO_4. F_1 was eluted with Buffer B plus 75 mM K_2SO_4. The fractions containing 80% of the activity were pooled, ammonium sulfate was added to 72% of saturation and the precipitate was stored at 4° C.

F_0F_1-ATPase was purified by sodium cholate/n-octyl β-D-glucopyranoside extraction (Rott and Nelson, 1981). Crude mitochondria were prepared as described above. Sodium cholate and n-octyl β-D-glucopyranoside were added to 0.5% and 1% respectively and the mixture was incubated for 20 minutes at 0° C. The suspension was centrifuged at 200000 X g for 30 minutes. Ammonium acetate was added to the supernatant to 37% saturation and the suspension was incubated at 0° C for 20 minutes. The precipitate was removed by centrifugation at 10000 X g for 10

minutes, and ammonium sulfate was added to the supernatant to 48% saturation. The suspension was incubated at 0° C for 20 minutes and precipitated material was collected by centrifugation at 10000 X g for 10 minutes. The pellet was resuspended at a protein concentration of 5-10 mg/ml in 30 mM Tris succinate (pH 6.5), 0.2% Triton, 0.1 mM ATP, 0.5 mM EDTA, and 0.1% acetone washed L-α-phosphatidylcholine vesicles (added as 40 mg vesicles/ml 80 mM Tricine pH 8.0). Vesicles bearing reconstituted F_0F_1 were purified by centrifugation on 7 to 30% sucrose gradients at 100000 X g for 24 hours at 4° C. Gradients were fractionated and ATPase activity appeared at 4-6 ml from the bottom of 12 ml gradients.

Assay of ATPase Activity and Determination of Steady State Kinetic Constants

ATP hydrolysis was measured at 30° C by a coupled assay (Ebel and Lardy, 1975) in a reaction cocktail containing 20 mM Tricine pH 8.0, 0.45 mM NADH, 2 mM PEP, 6.5 mM KCl, 2 mM $MgCl_2$, and 2 units/ml each of lactate dehydrogenase and pyruvate kinase. One control reaction containing the reaction cocktail and submitochondrial particles without ATP was run with every five experimental reactions. The rate in this control was subtracted from the experimental reactions to eliminate any rate contributed by direct oxidation of NADH by constituents of the submitochondrial particles.

Immunologic Characterization of Submitochondrial Particles

Submitochondrial particles were separated on 10% SDS-polyacrylamide gels (Laemmli, 1971) the F_1-ATPase α and/or β subunits were detected by western blot as described (Maniatis et al, 1989). Anitsera raised against the α subunit, the β subunit, and the entire yeast F_1 complex were provided by Dr. Alfred S. Lewin.

Results

Phenotypic Characterization of Mutant Yeasts

Six mutations were engineered into *ATP2* as described in Methods. These mutations were: H211N, H211D, H211K, H211I, H211A, and L203F. Sequenced restriction fragments containing the mutations were used to replace the corresponding restriction fragment in pβOK. Plasmid pβOK is a multicopy yeast shuttle vector which carries all the necessary information for the transcription of *ATP2* under the control of its natural promoter. The resulting plasmids were named for the mutation they carried: pN211, pD211, pK211, pI211, pA211, and pF203. The yeast strain AVY4-1 (*atp2::LEU2*) was transformed with each of these plasmids separately. This resulted in six mutant strains, each expressing a different mutated β subunit. The ability of the mutant strains to respire aerobically was assayed by observing the size of colonies formed on a nonfermentable substrate (Fig. 2.7). Of the six mutant strains, only βL203F and βH211N were capable of aerobic respiration. The other strains formed no colonies. The growth rate of βL203F was greater than βH211N but less than that of the wild type. Because

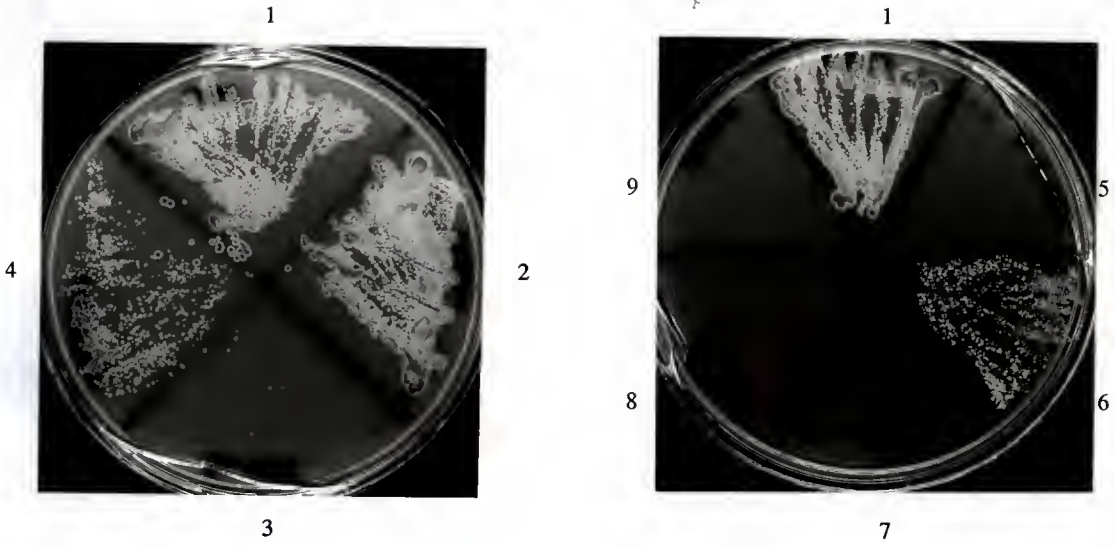

Fig. 2.7 Aerobic growth of wildtype and mutant yeasts. Wildtype and mutant yeasts were grown overnight in 3 ml of yeast minimal media. Samples from these cultures were streaked onto minimal plates with 2.5% glycerol and 2.5% ethanol as the sole carbon sources. Plates were incubated at 30° C for 3-5 days. 1: SEY103, 2: βOK, 3: AVY103, 4: βL203F, 5: βH211K, 6: βH211N, 7: βH211A, 8: βH211I, 9: βH211D.

these strains were capable of aerobic respiration, it was concluded that neither L203 nor H211 is required for catalysis by the wild type F_1-ATPase. Plasmid pβOK was used to transform AVY4-1 and the resulting strain was phenotypically wild type.

To assess whether the position 211 mutations were dominant or recessive, partial polyploids were constructed by transformation of SEY2102 (*ATP2*) with each of the shuttle vectors containing the position 211 mutations. SEY2102 is the parent strain of AVY4-1. The resulting strains contained a single copy of *ATP2* and multiple copies of *atp2* genes, and were therefore polyploid for the *ATP2* locus. These yeasts were then supplied with a nonfermentable carbon source and their growth was observed (Fig 2.8). The growth rate of each of the resulting strains was indistinguishable from the wild type, therefore each position 211 mutation is phenotypically recessive.

Five plasmids were constructed which carried doubly mutated *atp2* genes. The L203F mutation was paired with each of the position 211 mutations, so that each plasmid carried one copy of *atp2* which was mutated at two sites, positions 203 and 211. The resulting plasmids were named pL203F/H211N, pL203F/H211D etc. These plasmids were used to transform AVY4-1 and the capacity of the resulting strains to respire aerobically was assessed as above. Each of the doubly mutated strains was capable of aerobic respiration (Fig 2.9). L203F is therefore an intragenic suppressor of mutations H211D, H211K, H211I, and H211A. Kinetic data will be

Fig. 2.8 The position 211 mutations are recessive to wildtype. SEY2102 was transformed with each of five shuttle vectors carrying position 211 mutations. Single colonies were picked and grown as described in figure 2.7. Plates were incubated at 30o C for 3-5 days. 1: SEY103, 2: SEY/βH211D, 3: SEY/βH211I, 4: SEY/βH211A, 5: SEY/βH211N, 6: SEY/βH211K.

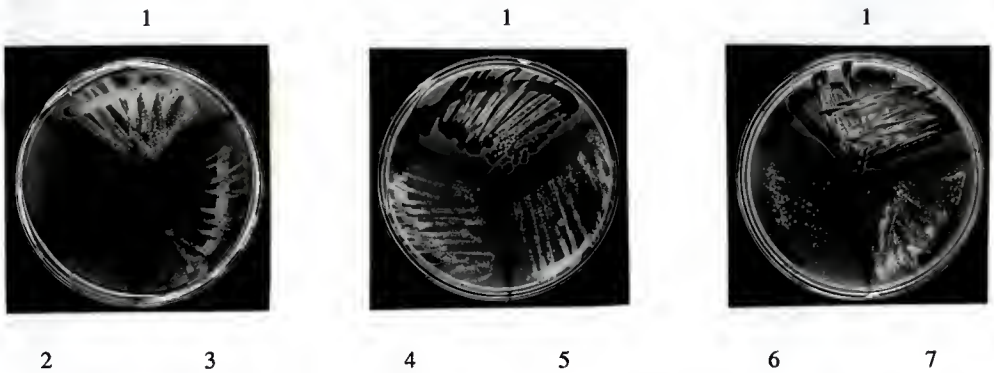

Fig. 2.9 Mutation L203F is an intragenic suppressor of the position 211 mutations. AVY4-1 was transformed with each of five shuttle vectors carrying doubly mutated copies of ATP2. Each plasmid carried the L203F mutation and on of the five position 211 mutations. Single colonies were picked and grown as described in Fig. 2.7. Plates were incubated at 30° C for 3-5 days. 1: SEY103, 2: AVY103, 3: βL203F/βH211K, 4 βL203F/βH211A, 5:βL203F/βH211I, 6: βL203F/βH211N, 7: βL203F/βH211D.

presented in the next chapter to show that L203F also suppresses H211N. These data are suggestive of a functional, and possibly structural, interaction between L203 and H211 of the β subunit.

Characterization of the Stability and Structure of the Mutant ATPases

In order to evaluate further the effects of mutations in the yeast F_1-ATPase β subunit, it was necessary to characterize the mutant ATPases with respect to their assembly, stability, and structure. Evaluation of several characteristics was required, these included subunit composition, stability of the enzyme over time, and stability under conditions of assay. The first step in these analyses is the purification of the enzymes.

Stability of chloroform extracts

Submitochondrial particles were prepared from each of the singly mutated strains, and each preparation showed oligomycin sensitive ATPase activity (see below). Chloroform extracts of the submitochondrial particles were then subjected to DEAE anion exchange chromatography as described in Methods. No significant ATPase activity was detected in any fraction of the eluates. The ATPase activities of the chloroform extracts were assayed before chromatography and were found to be unstable. The addition of 20% glycerol to submitochondrial particles prior to chloroform extraction did not stabilize the activities of the extracts. The addition of 20% glycerol to the ATPase assay likewise had no stabilizing effect. For each mutant strain tested, the specific activities of the chloroform extracts were greater than those of the submitochondrial particles,

but the rates of ATP hydrolysis decreased sharply after about 45 seconds of assay (Table 2.1). Specific activities of chloroform extracts from 2.25 minutes to 5 minutes of assay were reduced by 10-fold to 14-fold relative to values from the first 45 seconds. Addition of ADP to the reaction mixtures after 5 minutes resulted in a rapid loss of absorbance, thus showing that the observed decreases in rates were not due to oxidation of all the NADH in the reaction. These results were reproducible in separate experiments using material from the same extract. This is consistent with turnover-dependent, but not time-dependent, dissociation of F_1. The activities of the chloroform extracts were stable for at least 20 minutes, but were not stable enough to be purified by anion exchange chromatography which takes about 7 hours.

Stability of sodium cholate and octyl glucoside extracts

The sodium cholate and octyl-G-glucopyranoside extraction method of Rott and Nelson (1981) was used to reconstitute active wild type F_0F_1 complexes in phospholipid vesicles. Preparations of wild type ATPase reproducibly showed oligomycin-sensitive ATPase activity at 4-6 ml from the bottom of a 12 ml sucrose gradient. This is considered to be the gentlest purification procedure for mitochondrial ATPases. Nonetheless no ATPase activity was recovered from the mitochondria of any strain carrying substitutions at positions 211 or 203 using this protocol. These results suggest that mutations at position 211 destabilize the F_1 complex, as does the substitution of phenylalanine for leucine at position 203.

Table 2.1 Specific activities of chloroform extracted ATPases from mutant yeast strains.

	Specific Activity	
Strain	0-0.75 min.	2.25-5 min.
βH211D	10.9	1.1
βH211I	6.9	0.5
βH211A	13.5	1.3
βH211N	8.4	0.9
βH211K	5.2	0.4

Submitochondrial particles were adjusted to a pH of 6.25 extracted with 0.5 volumes of chloroform, and the ATPase activities of the extracts were determined by coupled assay as described in Methods. The concentration of ATP in the assay was 12 mM and the pH of the assay medium was 6.25. Specific activity is expressed in μmol X $min^{-1}mg^{-1}$ protein. Values shown for mutants are the average of three experiments. Wildtype values were in the range of 15-20 μmol X $min^{-1}mg^{-1}$ and did not decrease over the course of the assay.

Submitochondrial particles prepared from the mutant strains retain most of their ATPase activity after months of storage at -75° C, but separation of the F_1 portion from the membrane results in destabilization of the complexes. Association with membrane bound F_0 appears necessary to stabilize these enzymes.

Because the ATPases from the mutant strains could not be purified, all of the kinetic and physical characterizations in this study were performed on submitochondrial particles. Unless otherwise indicated, all statements regarding ATPase activities of the mutants therefore refer to activities of submitochondrial particles isolated from the mutant strains. For purposes of comparison, submitochondrial particles from the wild type were used as well, rather than pure F_1. The stability over time of the membrane bound ATPases was assayed as follows. Submitochondrial particles were incubated at 30° C and their ATPase activity at pH 8.0 was measured several times over 4 hours. Strains SEY103, βOK, and βH211I showed no loss of ATPase activity in this experiment, while strains , βH211D, βH211A, and βH211K displayed 84%, 71%, 70%, and 67% of their original activities after 4 hours at 30° C. ATPase activities during each five minute assay did not decrease during the assay period. This experiment was repeated with incubation of the submitochondrial particles at 0° C rather than 30° C, and no loss of activity was seen in any strain after 3 hours of incubation. In all experiments in this study in which ATPase activity is measured, the

submitochondrial particles were kept on ice for less than three hours prior to assay.

Immunological characterization of the mutant ATPases

In order to assess the subunit composition of the mutant ATPases a series of immunoblots were performed. Submitochondrial particles from wild type and mutant yeast were separated by SDS-PAGE, transferred to PVDF membranes and probed with antisera raised against either purifed F_1 α subunits or purified F_1. The results of these experiments can be summarized as follows. The quantity of α subunit present in submitochondrial particles from host strain AVY4-1 was influenced by the presence of the β subunit. For example, particles from strain βOK contained as much α subunit per unit protein as did particles from SEY103 (Fig. 2.10). The quantity of α subunit in particles from the mutant strains varied with the preparation, but was never more than about one third the amount seen in βOK or SEY103 (Fig. 2.11). The quantity of α subunit in AVY4-1 particles, which contained no detectable β subunit, was at least 10-fold less than in SEY103 or βOK particles (Fig. 2.12). The ratio of β to α subunits in the mutant strains and in βOK was slightly higher than that of SEY103 (Fig. 2.10). This may reflect the presence of unassembled β subunits due to the presence of multiple copies of the expression plasmid and subsequent overexpression. The quantities of α subunits detected in these assays allow an upper limit estimate of the amount of ATPase complexes which can be formed in the mutant strains under the growth conditions described.

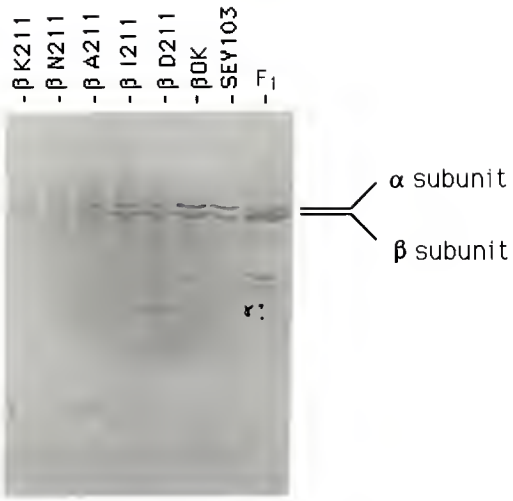

Fig. 2.10 Quantitation of α and β subunits in submitochondrial particles from mutant and wildtype yeasts and soluble yeast F_1-ATPase. Submitochondrial particles were solublized in SDS-PAGE sample buffer, and immunoblots were performed as described in Methods using antiserum raised against yeast F_1-ATPase. Lanes βK211-SEY103: 30 μg protein loaded per lane. Lane F1: 3 μg of pure yeast F_1-ATPase from strain SEY2102 loaded.

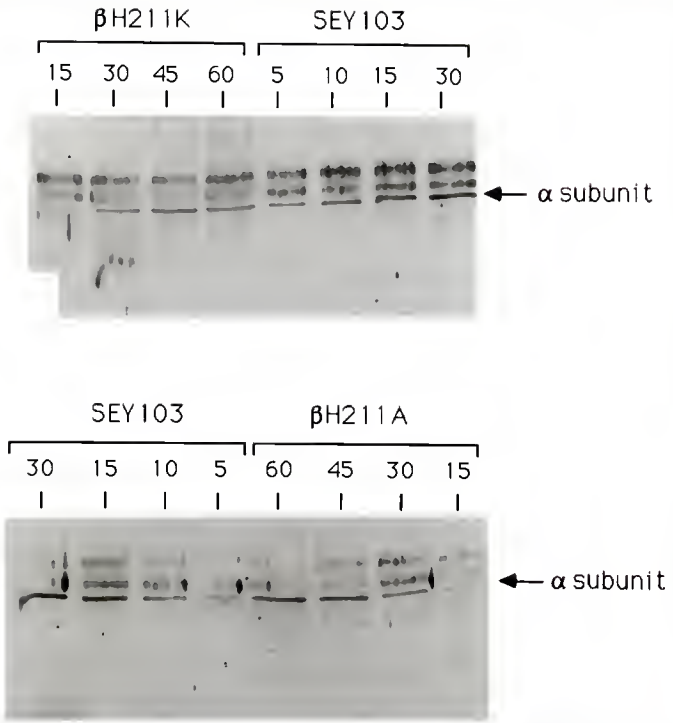

Fig. 2.11 Quantitation of the α subunit in submitochondrial particles from mutant and wildtype yeast. Submitochondrial particles were solublized in SDS-PAGE sample buffer and immunoblots were performed as described in Methods. Antiserum was specific for the α subunit. The amount of protein loaded in each lane is indicated in μg.

Fig. 2.12 Relative amounts of α subunit in yeast strains AVY4-1 and SEY103. Immunoblots of submitochondrial particle protein were performed as described in Methods using antiserum raised against yeast F_1-ATPase. The amount of protein loaded per lane is indicated in μg. The migration of the α and β subunits is shown.

The total ATPase complexes formed per unit mitochondrial protein in the strains carrying mutations at position 211 cannot be greater than one third of that in strains βOK and SEY103. The minor subunits were not detectable by the anti-F_1 antiserum in the widtype or mutant submitochondrial particles.

Oligomycin sensitivity of the mutant ATPases

The oligomycin sensitivity of the mutant ATPases was evaluated as an indirect test of ATPase assembly. Inhibition of mutant ATPases by oligomycin requires that oligomycin sensitivity conferring protein (OSCP) is assembled into the complex (Tzagoloff, 1970). Fig 2.13 shows the effects of oligomycin and methanol on ATP hydrolysis by submitochondrial particles from the wild type and mutant yeasts. Oligomycin was added to the reaction as a methanolic solution from a 2 mg/ml stock, and equivalent amounts of pure methanol were added to parallel reactions as a control. ATPase activity was measured at a substrate concentration of 6 mM. ATPase activities of the wild type and mutant strains were not significantly affected by methanol concentrations up to 2%. Maximal inhibition of the ATPases occurred at an oligomycin concentration of 20 μg/ml (this corresponds to a methanol concentration of 1%). At pH 8.0 ATPase activities of the mutants were inhibited by 30-50%, while SEY103 and βOK strains were inhibited by 93.3% and 93.8% respectively. The experiment was repeated at pH 6.5 with little change in the extent of inhibition of the mutant ATPases, while inhibition of βOK by 20 μg/ml oligomycin decreased to 64%. The

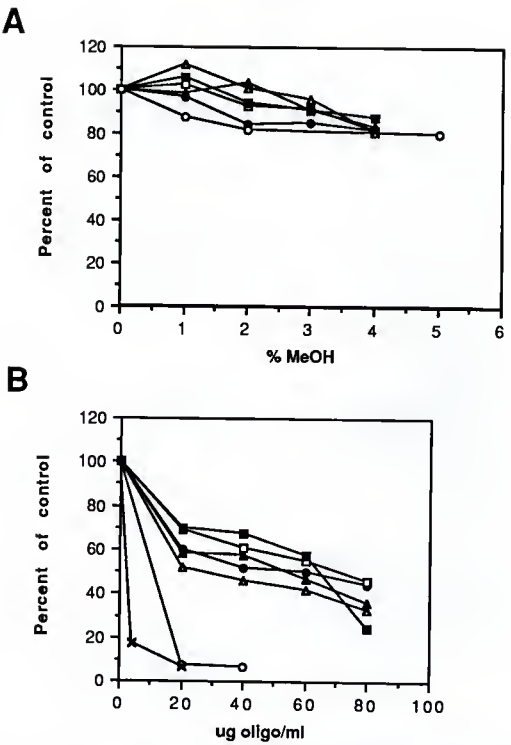

Fig. 2.13 The sensitivity of wild type and mutant F1-ATPases to oligomycin. Panel A: the effect of methanol on ATP hydrolysis. Panel B: the effect of oligomycin on ATP hydrolysis. Oligomycin was added to the concentrations shown from a 2 mg/ml methanolic stock solution. The amount of methanol in a reaction containing 20 μg/ml oligomycin is 1% v/v. Rates of ATP hydrolysis were measured as described in Methods and are expressed as percentages of the rates measured in the absence of methanol or oligomycin. Rates are averages of 2-3 measurements Open circles: SEY103, closed circles: βH211D, open squares: βH211I, closed squares: βH211A, open triangles: βH211N, closed triangles: βH211K, X: βOK.

reduced sensitivity to oligomycin of the ATPases from the mutant strains is suggestive of structural perturbation in these complexes, however it must be concluded that the oligomycin sensitivity conferral protein is assembled into each complex.

The effect of organic solvents on the mutant ATPases

In the course of measuring oligomycin sensitivity, it was noted that a methanol concentration of 5% caused a slight decrease in the rate of ATP hydrolysis by the mutant enzymes. This inhibition increased when the methanol concentration was adjusted to 10%. It was proposed that this apparent inhibitory effect was due to the destabilization of the enzyme complexes by the increasing nonpolar character of the solvent. In order to assess further the stability of the mutant enzymes, their activities in the presence of several organic solvents was investigated. We chose to use 2-propanol, ethanol, methanol, and dimethyl sulfoxide on the basis of their varying degrees of polarity. Figs. 2.14-2.17 show the effects of these solvents on the activities of the wild type and mutant ATPases. The general trend for the effect of 2-propanol is similar to that for ethanol, although 2-propanol does not seem to have as negative an effect at 1% and 2%, and in the cases of βH211A and βH211K it is stimulatory at these concentrations. Methanol had little effect on ATP hydrolysis by the mutants or the wild type. Dimethyl sulfoxide stimulated the activities of the mutant ATPases but inhibited the wild type at concentrations of 5% and 10%. The stimulatory effect of

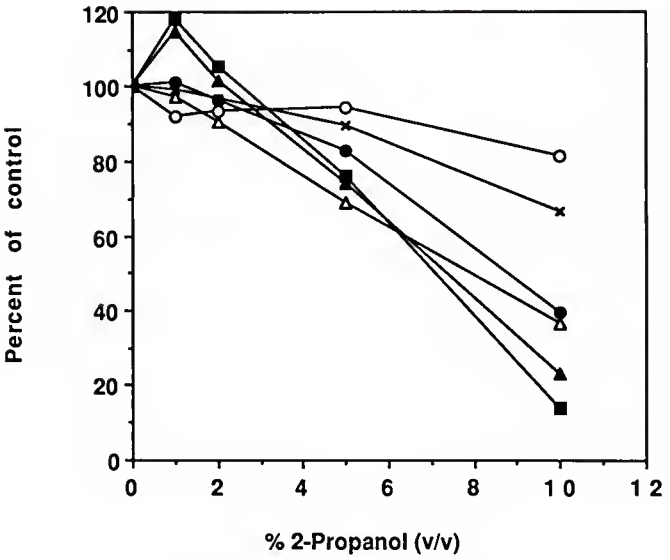

Fig. 2.14 The effect of 2-propanol on ATP hydrolysis by mutant and wild type ATPases. Rates of ATP hydrolysis in the presence of 2-propanol are expressed as percentages of the rates determined in the absence of ethanol and are averages of 2 or 3 determinations. ATP hydrolysis was measured by coupled assay as described in Methods. Reactions included 12 mM ATP and 150 μg submitochondrial particles/ml, except for SEY103 for which 20 μg/ml were used. Open circles: SEY103, closed circles: βH211D, open squares: βH211I, closed squares: βH211A, open triangles: βH211N, closed triangles: βH211K, X: βL203F.

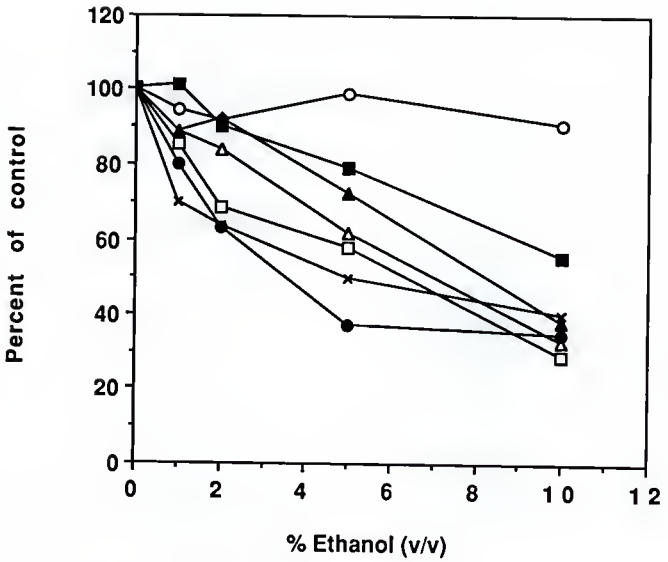

Fig. 2.15 The effect of ethanol on ATP hydrolysis by mutant and wild type ATPases. The experiment was performed precisely as described in Fig. 2.14. Rates of ATP hydrolysis in the presence of ethanol are expressed as percentages of the rates determined in the absence of ethanol and are averages of 2 or 3 determinations. Open circles: SEY103, closed circles: βH211D, open squares: βH211I, closed squares: βH211A, open triangles: βH211N, closed triangles: βH211K, X: βL203F.

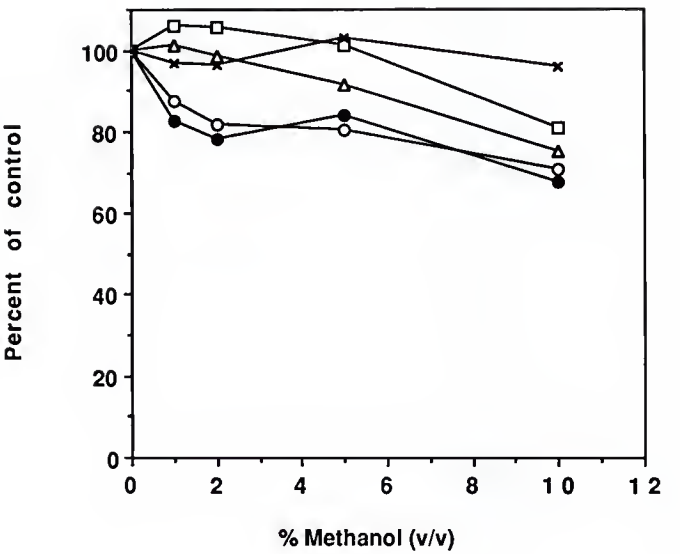

Fig. 2.16 The effect of methanol on ATP hydrolysis by mutant and wild type ATPases. The experiment was performed precisely as described in Fig. 2.14. Rates of ATP hydrolysis in the presence of methanol are expressed as percentages of the rates determined in the absence of ethanol and are averages of 2 or 3 determinations. Open circles: SEY103, closed circles: βH211D, open squares: βH211I, open triangles: βH211N, X: βL203F.

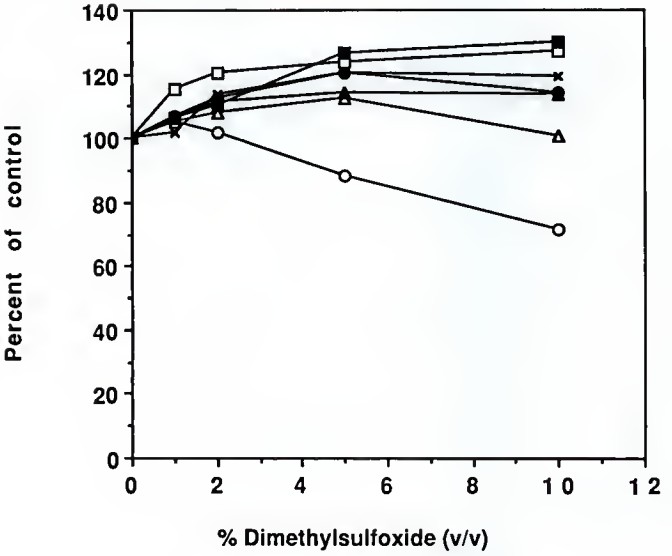

Fig. 2.17 The effect of dimethylsulfoxide on ATP hydrolysis by mutant and wild type ATPases. The experiment was performed precisely as described in Fig. 2.14. Rates of ATP hydrolysis in the presence of dimethylsulfoxide are expressed as percentages of the rates determined in the absence of dimethylsulfoxide and are averages of 2 or 3 determinations. Open circles: SEY103, closed circles: βH211D, open squares: βH211I, closed squares: βH211A, open triangles: βH211N, closed triangles: βH211K, X: βL203F.

dimethyl sulfoxide on the mutants was greatest at 5% and 10%. The effect reached a plateau for βH211A, βH211I, βH211I, and βL203F at 5%. Stimulation decreased at 10% for βH211N and βH211D.

Discussion

The roles of positions L203 and H211 of the yeast F_1 ATPase β subunit were investigated by substituting Phe for Leu at position 203 and Asn, Lys, Asp, Ile, and Ala for His at position 211. Mutagenesis was performed in a plasmid which was specifically modified to facilitate mutagenesis of *ATP2*. Sequenced restriction fragments containing the mutations were used as cassettes to replace the corresponding restriction fragments in wild type copies of *ATP2* carried on the yeast shuttle vector pβOK-H. The resulting plasmids were used to transform yeast strains AVY4-1 (*atp2::LEU2*) and SEY2102 (*ATP2*).

H211 and L203 are not Required for Aerobic Respiration or Catalysis

Of the five amino acids substituted for histidine, asparagine is the best structural mimic. The results in Figure 2.7 show that strain βH211N is the only position 211 mutant capable of aerobic respiration. This suggests that a positive charge on H211 is not involved in the wild type catalytic mechanism. The asparaginyl amide nitrogen of H211N may occupy nearly the same spatial location as the number 1 nitrogen of the imidazole ring of H211. It is possible that position 211 may be involved in hydrogen bonding, and that a histidine at this residue is better positioned to achieve this than asparagine. A hydrogen bond at position 211 might be required

for either assembly or structural stabilization. None of the other position 211 mutants respires aerobically, suggesting that either the sidechain shape or the hydrogen bonding potential of this residue is critical for enzyme function. Tian et al. (1988) have performed site-directed mutagenesis on the corresponding histidine of adenylate kinase (H36). As discussed above, the primary result of the amino acid substitutions was the destabilization of the enzyme. The authors offered three explanations for this based on the three dimensional models available. They proposed that H36 either hydrogen bonded with C25, formed a charge-transfer complex with C25 and D93, or protected C25 by shielding it while also forming a hydrogen bond with D93. In each case the number 3 nitrogen of the imidazole group is predicted to be involved in an electrostatic interaction. In this context a substitution of glutamine for H211 may have been more instructive than the substitution of asparagine for H211. In any case, the results suggest that the shape of the residue at position 211 is more important than its hydrophobic character, and the ability to form a hydrogen bond may also be important.

H211 and L203 are Required for the Proper Assembly of the F_1-ATPase Complex

Phenotypic analyses

Before meaningful kinetic analyses can be performed on the mutant enzymes, the issue of assembly of the F_0F_1 complex in the mutant strains must be addressed. Much of the data presented in this chapter is pertinent to this point. The recessive nature of the

position 211 mutations was demonstrated by transforming yeast strain SEY2102 (*ATP2*) with multi-copy yeast shuttle vectors carrying *atp2* mutated at codon 211. This resulted in yeast which were polyploid for the *ATP2* locus, but which carried only one wild type allele. The fact that these yeast were phenotypically wild type raises the question of whether or not the mutant β subunits assemble with wild type subunits to form hybrid F_1 complexes. Noumi et al (1987) and Rao and Senior(1987) reported the formation of *E. coli* F_1 hybrid complexes using mutated β and α subunits respectively. Rao and Senior generated *uncA*$^-$ *E. coli* strains incapable of aerobic respiration. They then purified F_1, dissociated it, and reassociated the complexes in the presence of varying amounts of wild type α subunit. The reassembled complexes containing both wild type and mutated α subunits had diminished catalytic activity relative to the wild type. Noumi et al. reported similar results with mutated β subunits. The formation of hybrid complexes in the yeast system should also result in a decreased catalytic capacity which might be manifest as a decreased growth rate on a nonfermentable carbon source. Based on growth rates for the polyploid yeast strains in this study, it seems likely that the mutated β subunits are preferentially excluded from the F_1 complex in favor of the available wild type subunits. This might be expected if the mutated β subunits were not refolded correctly after transport into the mitochondrial matrix. It is possible that the mutations affect the interaction of the β subunits with $hsp60^{MIF4}$ or with the α subunit prior to assembly. This raises

the questions of how well the mutant β subunits are assembled into the F_0F_1 complex in the absence of wild type β subunits, and what pleiotropic effects do the mutations have on the assembly of the other subunits?

Immunologic characterization

Because the mutant ATPases could not be purified, analysis of their subunit compositions is technically difficult. Western blot analyses of submitochondrial particles from the mutant strains can show the identities and relative quantities of subunits present in the mitochondria, but may yield misleading information regarding assembly. Immunoprecipitation of the complexes with antisera directed to a single subunit may also be misleading because of the instability of the mutant complexes. With these problems in mind, western blot analyses using polyclonal antisera directed against either the α subunit or the whole F_1 complex were carried out on submitochondrial particles from the mutant and wild type strains. The results show that the mutations at position 211 affect the assembly of the F_1 complex. The nature of this effect is not known beyond the fact that the mutant strains assemble no more than one third as much F_1 as the wild type. It seems likely that the rate of either assembly or disassembly or both has been affected such that the equilibrium amount of complexes formed is less than in the wild type. This would be consistent with the exclusion of the mutated subunits from the ATPases formed by the heterozygous partial polyploid strains. The issue of subunit composition of the mutant

ATPases with respect to the minor subunits was not resolved by immunological analysis and will be addressed below.

The observed influence of the β subunit on the amount of α subunit detected in submitochondrial particles is in apparent contradiction to the results of Takeda et al. (1985) who found that the absence of the β subunit had no effect on the import of the α subunit into the mitochondria of MDY2102 (an *atp2::Leu2* strain derived from SEY2102). These authors immunoprecipitated α and β subunits from detergent extracts of whole mitochondria. The discrepancy in results may be due to the use of submitochondrial particles in this study, rather than whole mitochondria. It is possible that after disruption of the mitochondria, unassembled α subunits were released and not recovered in the submitochondrial particle sediment.

Assembly of minor subunits

The oligomycin sensitivity conferral protein forms part of the stalk region between F_1 and F_0. Uh et al. (1990) have shown that the presence of OSCP is required for the binding of F_1 to F_0. That this protein assembles into the mutant ATPase complexes is obvious because they are each sensitive to oligomycin. However, the degree of sensitivity to oligomycin is much reduced relative to the wild type. This is difficult to interpret unambiguously, but is suggestive of structural perturbation due to the β subunit mutations. The assembly of the ε subunit into the complexes can be inferred indirectly since it has been shown (Guelin et al., 1993) that ε null

mutants have no oligomycin sensitive ATPase activity. The presence of the γ and δ subunits has not been demonstrated. In the *E. coli* system, all eight subunits of F_0F_1 are required for ATP synthase activity (Senior 1990). This fact provides some support for the contention that the F_0F_1 moiety of strain βH211N is fully assembled. An argument for the full assembly of the mutant F_1 complexes based on kinetic data will be presented in the next chapter.

The Effect of Mutations at Positions 211 and 203 on the Stability of the F_1 Complex

Several lines of evidence suggest that H211 and L203 of the β subunit are important for the structural integrity of the F_1 complex. Attempts to purify ATPases from the mutant strains using techniques which involved either permanent or temporary removal of the F_1 portion of the enzyme from membrane bound F_0 failed. On the other hand, activities of submitochondrial particles from the mutant strains were stable after months of storage at -75°C. This suggests that alteration of either position 211 or 203 destabilizes the F_1 complex in such a way that it dissociates upon removal from F_0. Measurement of the activities of chloroform extracts of submitochondrial particles indicated that dissociation of the ATPases is either turnover dependent or is at least facilitated by catalysis (Table 2). Dissociation probably also occurs over time, but the activities of the chloroform extracts were stable for at least twenty minutes in the absence of MgATP.

The instability of the mutant ATPases was also demonstrated by the effects of ethanol and 2-propanol on catalysis by submitochondrial particles (Figs. 2.15 and 2.16). It was proposed that if the mutant ATPases were not folded entirely correctly, then exposure to organic solvents might cause further structural perturbation resulting in decreased activity. Ethanol and 2-propanol inhibited ATP hydrolysis by the mutant enzymes at concentrations which had little effect on the wild type ATPase. Inhibition increased with the concentration of ethanol or 2-propanol in the assay. Methanol had little effect on catalysis by ATPases from mutant or wild type strains at the concentrations studied. This is consistent with inhibition being due to increased nonpolarity of the assay solution.

Taken together, the reduced sensitivity of the mutant ATPases to oligomycin, the inhibitory effects of ethanol and 2-propanol, and the apparent instability of the altered F_1 complexes when separated from the membrane, provide strong evidence that H211 is important for the stabilization of the F_1 complex. Replacement of histidine at position 211 with amino acids of varying characteristics i.e. an acid, a base, hydrophobic residues, or a polar isosteric replacement results in destabilization of the complex. Replacement of leucine at position 203 with phenylalanine apparently causes similar effects.

The identification of L203F as an intragenic suppressor of the position 211 mutations was an unexpected result which bears directly on the question of the structural and functional interaction

of L203 and H211. There are several instances in the study of F_0F_1-ATPase alone in which intragenic or extragenic supressor mutations have been interpreted as evidence of structural or functional interaction between the positions at which the mutations occur (Kumamoto and Simoni 1986, 1987, Cain and Simoni, 1988, Nakamoto et al., 1993). As discussed above, high resolution structures of adenylate kinase have been used as a framework for proposing a structure for the *E. coli* F_1-ATPase β subunit nucleotide binding site (Duncan et al.,1986). In such a model L203 would reside near the end of an α helix which follows a glycine rich loop. After the C-terminus of the helix there is a hairpin turn in the peptide backbone which gives rise to the proposed hydrophobic cleft with which the adenine or ribose moiety is associated. In this model L203 is situated on the opposite side of the cleft from H211 (Figs. 2.2 and 2.3). It is conceivable that the sidechains from positions 203 and 211 interact and that substitution for either of them distorts the conformation of the complex in a similar manner. Each of the amino acids which were substituted at position 211 takes up less volume than histidine. On the other hand, phenylalanine is bulkier than the leucine at position 203. One simple explanation for the suppression of the position 211 mutations by L203F is that positions 203 and 211 interact physically. When the side chain bulk at position 211 is lessened by substitution of a smaller amino acid, the interaction does not occur. An increase in side chain bulk at position 203 then

reestablishes the interaction but not to the same extent as in the wild type.

Another speculative interpretation would be that the side chains at position 203 and 211 do not interact physically, but instead share a functional role. For example, both sidechains might interact with the adenine moiety of ATP as it associates with the hydrophobic cleft. Substitution of a smaller amino acid for histidine at position 211 could decrease the interaction of this residue with adenine. Insertion of a phenylalanine at position 203 might alleviate the decrease in hydrophobic character of the cleft, or it might simply "push" the adenine closer to position 211. A similar scenario could be envisioned if the role of H211 was to stabilize the nucleotide binding site by hydrogen bonding with the another residue analogous to D93 of adenylate kinase. Removal of the hydrogen bonding group at position 211 might interfere with the stabilization of the nucleotide binding site. Phenylalanine at position 203 might partially suppress the binding defect by increasing the hydrophobic nature of the cleft. This idea may be the most consistent with the results in that the H211N is the least deleterious mutation. If phenylalanine at position 203 alters the local structure of the protein so that the asparaginyl amide nitrogen can reestablish a stabilizing hydrogen bond, the original defect might be suppressed.

Alternatively, the suppression might be explained by a mechanism which does not require the interaction of residues 203 and 211. The L203F mutation may alter the structure of the β

subunit in such a way that further mutations at position 211 have little effect on the function of the β subunit. This can be envisioned as follows. While H211 is not required for catalysis, it may help maintain the structure of the active site. Single mutations at position 211 may result in interference with the catalytic mechanism, resulting in the phenotype of the position 211 mutants. A situation can be imagined where the mutation at position 203 alters the local structure of the protein such that position 211 is removed from its normal space in the tertiary structure. Position 211 would then be unavailable to perform its stabilization role. This results in the phenotype of L203F. Suppression of the position 211 mutations may result from their removal by the mutation at position 203. Position 211 would not fulfill its role in structural stabilization, but neither would it cause interference with the catalytic mechanism. A phenotype similar to the L203F mutant would then be expected.

Each of these explanations is highly speculative and, unfortunately, difficult to test. What is clear from the results of this chapter is that H211 is not required for the chemistry of catalysis in the yeast F_1-ATPase. The same can be said of L203. H211 and L203 are each important for the structural stability of the enzyme as evidenced by the failure to purify enzymes with mutations at these residues. Mutations at H211 also have a negative effect on the assembly of the ATPase complex. This is probably due to a role in the folding of the β subunit. The data are also consistent with a functional and/or structural interaction between L203 and H211.

This would in turn be consistent with the three dimensional model for the nucleotide binding site proposed by Duncan et al. One further interesting note is the report of Rao et al. (1988) which showed that the *E. coli* F_1-ATPase could not be reassembled from pure subunits unless the β subunits had bound ATP. If the mutated β subunits do not fold correctly after transport into the mitochondria, then they might bind ATP poorly and assemble into the complex more slowly than usual. This would be consistent with the relatively low amounts of ATPase detected in the mutant strains.

Although the mutations at position 211 destabilize the F_1 complex, the membrane-bound enzymes appear to be stable in the absence of organic solvents. Submitochondrial particles from each of the mutant strains retain most of their activity after months of storage at -75° C, and incubation for several hours at 0° or 30° C results in little or no loss of activity. The stability of the membrane-bound ATPases provides the opportunity to characterize them kinetically. Kinetic studies may yield information concerning the possible roles of H211 in substrate binding, structural stabilization, and any indirect contribution to the catalytic mechanism.

CHAPTER 3

KINETIC CHARACTERIZATION OF MUTANT ATPASES

Introduction

The results of chapter 2 show that H211 of the yeast F_1-ATPase β subunit is not required for catalysis but is necessary for the structural stabilization of the F_1 complex. This does not exclude a role in substrate binding as has been proposed for the homologous residue of the rat liver F_1 β subunit (Garboczi et al., 1988). In order to characterize further the role of H211 of the yeast F_1 β subunit, kinetic analyses of the mutant ATPases were undertaken. First, K_m values for ATP hydrolysis by the mutant enzymes were determined. This was done to evaluate the contribution of H211 to substrate binding. Second, the pH optima for ATP hydrolysis, and the pK_a values of the groups involved in catalysis were determined in order to assess the influence of position 211 on the catalytic mechanism. Finally, the substrate preferences of the wildtype and mutant enzymes were qualitatively determined in order to gain insight into structures of the active sites of the mutant enzymes. In an additional experiment, the contribution of the 2' hydroxyl group of ATP to the wildtype catalytic mechanism was investigated by comparing the kinetic constants of ATP and dATP hydrolysis.

Methods

Determination of Kinetic Constants and pK_a Values

K_m and V_{max} for ATP hydrolysis were determined by analysis of v vs [S] plots. Initial velocities were measured at ten different substrate concentrations. Each velocity was the average of 3-6 measurements. The Enz-Fitter program was used to fit a curve to the data and to calculate K_m and V_{max} using the Michaelis-Menten equation.

The buffer systems used for determination of pH profiles were 50 mM each MES, BES, and BICINE for pH 5.5-9.0, and 50 mM each PIPES, TRICINE, and CHES for pH 6.25-10.0. At pH values of 5.5 and 6.0 the characteristic lag of the pyruvate kinase-lactate dehydrogenase coupling system became pronounced. To alleviate this problem, 8 units of each enzyme were added to each ml of reaction cocktail immediately before each assay. This ensured the measurement of initial rates at these pH values. pK_a values for ionizing groups involved in catalysis were calculated using the BELL program (Cleland, 1979). Stability of ATPases at extremes of pH was confirmed by incubation of submitochondrial particles at pH 5.5 or 9.0 for 30 minutes at 4° C and subsequent assay of activity at 12 mM ATP and pH 8.0. The stability of the submitochondrial particle ATPases used in the determination of pK_a was further tested by incubation in the assay mixture at either pH 5.5 or pH 8.5 and 30° C for the standard assay length (5 minutes), pH was then adjusted to 6.5 and activity was compared to that of particles which had been

incubated at pH 6.5 for 5 minutes at 30° C. No loss of activity as a result of incubation at pH 5.5 or 8.5 was detected by this assay.

Results

Measurement of K_m Values for ATP Hydrolysis by the Mutant Enzymes

Submitochondrial particles were prepared from each of the mutant strains and K_m values for ATP hydrolysis were determined (Table 3.1). K_m values for mutant strains βH211D, βH211I, βH211A, and βH211K are about 5-fold greater than the wild type. The K_m value for βH211N is about 3.5-fold greater than wild type. This indicates that H211 of the β subunit contributes to substrate binding either directly or indirectly. The K_m value for βL203F was similar to wild type. This suggests that position 203 is more likely to be involved in maintenance of the tertiary structure of the β subunit than in a binding interaction with the substrate. K_m values for the doubly mutated enzymes were in a range from 0.2 to 0.6 mM. In addition to the mutations at positions 211 and 203, a substitution of serine for cysteine at position 32 was also constructed. The aerobic respiration phenotype and K_m for ATP hydrolysis of βC32S were the same as wild type and this mutation was not studied further.

The mutant ATPases have been shown to be unstable when removed from F_0, have diminished sensitivity to oligomycin, assemble to a lesser extent than does the wild type enzyme, and have elevated K_m values relative to the wild type. These results raise the question of whether the mutant enzymes utilize the same

Table 3.1
K_m values for ATP Hydrolysis by Mutant ATPases.

Strain	K_m (mM)	K_m when paired with L203F (mM)
SEY2102	0.2-0.4	
βOK	0.2-0.4	
βC32S	0.33	
βL203F	0.17 ± 0.02	
βH211N	0.89 ± 0.12	0.11 ± 0.05
βH211K	1.10 ± 0.14	0.33 ± 0.06
βH211A	1.35 ± 0.18	0.19 ± 0.06
βH211D	1.37 ± 0.13	0.36 ± 0.14
βH211I	1.77 ± 0.25	0.21 ± 0.08

Initial velocities were measured by coupled assay as described in Materials and Methods.

reaction mechanism as the wild type enzyme. In order for comparisons of kinetic data between wild type and mutant strains to be meaningful, the same catalytic mechanism must operate in the both the mutant and wild type enzymes. Two characteristics of the F_1 catalytic
mechanism are negative cooperativity of substrate binding and positive catalytic cooperativity. In the absence of positive cooperativity catalysis occurs through a uni-site mechanism. The rates characteristic of this mechanism are too slow to be measured practically with the coupled assay system, therefore this characteristic of the mechanism is unchanged. Negative cooperativity of substrate binding is results in a curvature of double reciprocal plots of initial velocity data. This curvature was present in the double reciprocal plots of each of the mutant ATPases (Fig. 3.1). Another characteristic of the wild type catalytic mechanism is stimulation by oxyanions such as bicarbonate. Submitochondrial particles from strain βH211N showed bicarbonate stimulation (Fig. 3.1), the other mutants were not done. These data are not suggestive of the existence of an alternative catalytic mechanism in the mutant ATPases, but such a mechanism cannot be ruled out at this point. As discussed in the Chapters 1 and 2, the F_0F_1-ATPase has a complex reaction mechanism which depends on the interaction of many different subunits. The fact that the characteristics of negative cooperativity of substrate binding and positive catalytic

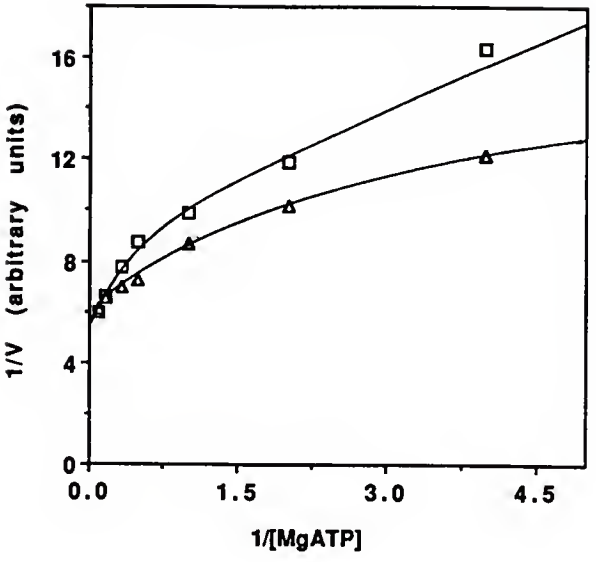

Fig. 3.1 Bicarbonate stimulation of F_0F_1-ATPase from strain βH211N. Initial velocity was measured at seven different substrate concentrations by coupled assay at pH 8.0. Submitochondrial particles were used at a concentration of 75 μg/ml. Squares: no bicarbonate added, triangles: bicarbonate added to 20 mM.

cooperativity are retained by the mutant enzymes suggests that these complexes do assemble all of the F_1 subunits.

Determination of pH Optima of the Mutant Enzymes

Each of the position 211 mutants retains ATPase activity. This implies that H211 is not required in the chemistry of catalysis. However, the increase in K_m for ATP hydrolysis in each mutant strain suggests that H211 may be involved in nucleotide binding or in maintenance of the active site structure. Such a role could depend on the ionization state of H211. To investigate this possibility, the pH optima for ATP hydrolysis by the wild type and βH211K strains were determined by measuring velocity at 12 mM substrate for several values of pH. It was predicted that if the positive charge of histidine has a role in substrate binding, then the substitution of lysine at position 211 might increase the pH optimum of the enzyme or broaden the optimum toward a greater pH value. Contrary to this prediction, it was found that the activity of βH211K decreased rapidly above pH 8.0 (Fig 3.2). There was no measurable activity for either βH211K or wild type at pH 10.0. On the other hand, at pH 6.75 the activity of βH211K increased to 435% of its value at pH 8.0. The pH optima of the ATPases from the other position 211 mutants as well as βL203F were then determined (Fig. 3.3). In each case the pH optimum for ATP hydrolysis was in the range of 6.0 to 6.5, and in three cases, βH211I, βH211N, and βH211D, the velocities at pH values 6.0 and 6.5 were greater than those of the wild type. It should be noted that the concentration of F_0F_1-ATPase in the submitochondrial

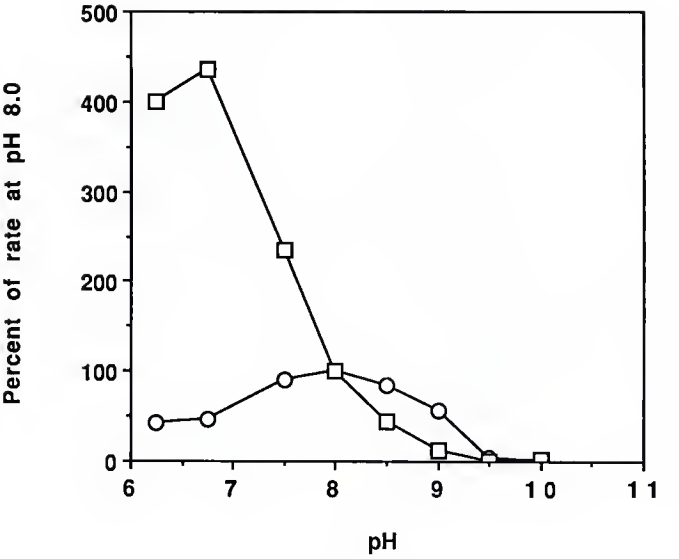

Fig. 3.2 The influence of pH on ATPase activities of submitochondrial particles from wild type and strain βH211K. Rates of ATP hydrolysis were determined by coupled assay at 12 mM ATP at pH values of 6.25, 6.75, 7.5, 8.0, 9.0, 9.5, and 10.0. Rates are expressed as a percentage of the rate measured at pH 8.0. Squares: βH211K (150 μg/ml), circles: SEY103 (20 μg/ml).

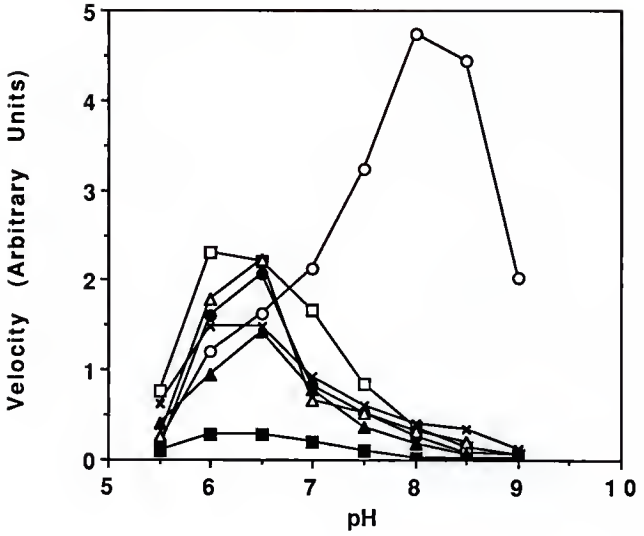

Fig. 3.3 The influence of pH on ATP hydrolysis by submitochondrial particles from mutant and wild type yeasts. Rates of ATP hydrolysis were measured by coupled assay at pH values 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The values shown for strains βH211A, βH211I and βL203F were determined at 12 mM ATP and are the averages of at least 3 measurements. All other values are V_{max} measurements determined as described in Materials and Methods. The concentration of submitochondrial particles from mutant strains was 150 μg/ml, except for βH211I, βH211N and βH211D at pH values of 6.0 and 6.5 where only 75 mg/ml was used. The concentration of SEY103 submitochondrial particles was 20 μg/ml.Open circles: SEY103, closed circles: βH211D, open squares: βH211I, closed squares: βH211A, open triangles: βH211N, closed triangles: βH211K, X: βL203F.

particle preparations is not known, therefore comparisons of V_{max} values between mutants are not appropriate. However, as shown in Chapter 2, it is known that the mutant strains assemble no more than one third as much ATPase per unit protein than the wild type, hence it is valid to conclude that ATPases from strains βH211I, βH211N, and βH211D have greater V_{max} values than the wild type at pH values from 6.0 to 6.5. There are several possible explanations for these data. Substitutions at position 211 may alter the structure of the active site such that hydrolysis occurs by a new catalytic mechanism with a lower pH optimum. Alternatively, the reaction may proceed through the wild type mechanism by virtue of ionization-dependent structural changes which restore or approximate the wild type conformation at low pH. It is unlikely that the change in pH optimum is an artifact caused by disassembly of the F_1 complex at pH values greater than 6.5 (see control experiments in Methods). No loss of activity was detected during the course of the assay by any submitochondrial particle preparation at any pH. By comparison, the unstable chloroform extracts of the mutant ATPases began to lose activity after 1 minute of assay (Table 2.1).

Determination of pKa Values for the Groups Involved in Catalysis

To investigate further the role of ionization at position 211, and to address the possibility of an alternative mechanism, V_{max} and K_m were determined at several different pH values for the ATPase activities of the wild type, βH211K, βH211N, and βH211D strains. The

data were plotted as log V_{max}/K_m vs pH and in each case showed a pattern typical of acid-base catalysis (Fig. 3.4). For the wild type enzyme, values of 5.4 $\pm$ 0.03 and 9.7 $\pm$ 0.09 were calculated for pK_{a1} and pK_{a2}, respectively (Table 3.2). The experiment was repeated with purified wild type F_1 and comparable values were obtained (5.3 $\pm$ .05 and 9.7 $\pm$ 0.10). The enzymes from βH211K, βH211N, and βD211 have pK_a values in the ranges of 5.3-5.5 (pK_{a1}) and 8-8.4 (pK_{a2}). The replacement of histidine therefore has no large effect on the group responsible for pK_{a1}, but does result in a substantial decrease in the pK_{a2} value. This is consistent with structural perturbation of a portion of the active site. The effect of this structural change could be either to alter the pK_a of the existing basic group, or to introduce a different basic group into the mechanism. The decreased pK_{a2} values of the mutant ATPases are not artifacts caused by disassembly of the ATPases at basic pH. The stability of the submitochondrial particle ATPases used in the determination of pK_a was confirmed by incubation in the assay mixture at either pH 5.5 or pH 8.5 and 30° C for the standard assay length (5 minutes), pH was then adjusted to 6.5 and activity was compared to that of particles which had been incubated at pH 6.5 for 5 minutes at 30° C. No loss of activity as a result of incubation at pH 5.5 or 8.5 was detected by this assay.

Hydrolysis of Alternative Substrates

The effect of mutations at H211 on substrate specificity was qualitatively assessed by comparing the rates of hydrolysis of several different nucleoside triphosphates by submitochondrial

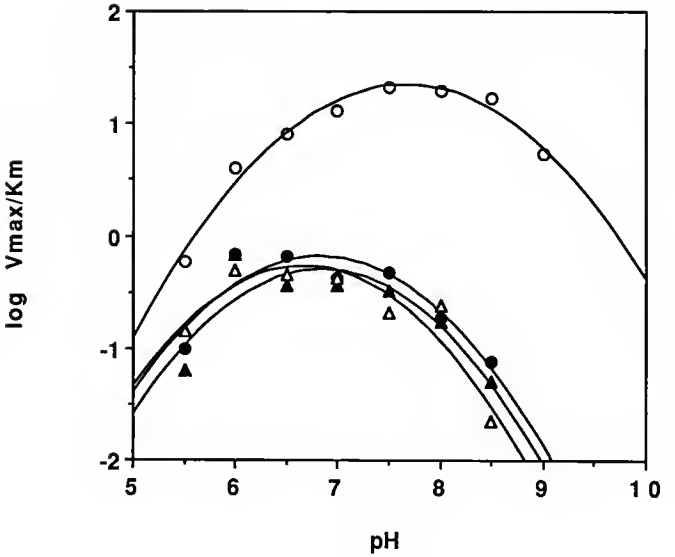

Fig. 3.4 Plots of V_{max}/K_m for ATP hydrolysis by mutant and wild type ATPases as a function of pH. Kinetic constants for ATP hydrolysis were determined as described in Materials and Methods.

Table 3.2 Values for pK_a of ionizable groups involved in the catalytic mechanism of wildtype and mutant ATPases.

Strain	SEY103	SEY103 F_1	βH211N	βH211D	βH211K
pK_{a1}	5.43 ± 0.03	5.3 ± 0.03	5.33 ± 0.34	5.38 ± 0.15	5.52 ± 0.18
pK_{a2}	9.71 ± 0.09	9.7 ± 0.09	7.96 ± 0.17	8.41 ± 0.11	8.29 ± 0.15

V_{max} and K_m were determined at pH values of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 as described in Methods and plotted as shown in Fig. 3.4. Values for pK_a and standard error were determined using the BELL program (Cleland, 1979) which fits the data to the equation

$$\text{Log } V_{max}/K_m = \text{Log}[C/(1+H/K_a + K_b/H)]$$

where C is a constant, H is the proton concentration, and K_a and K_b are the disociation constants of the general acid and general base, respectively. SEY103 F1 refers to purified, soluble F_1-ATPase, submitochondrial particles were used for all other measurements.

particles from wild type and mutant yeasts. The substrates examined were dATP, GTP, ITP, XTP, CTP, and UTP. Rates of hydrolysis were determined at pH values of 6.25 and 8.0 for a substrate concentration of 5.5 mM, and substrates were ranked in decreasing order of hydrolysis rate by each enzyme at each pH (Table 3.3). The results allow several observations. For each substrate tested, the difference in rate as a function of pH is not nearly as dramatic as the one seen when ATP is the substrate. CTP is the only substrate for which the velocities of the mutants are significantly greater at pH 6.25 than at pH 8.0. The sole exception to this is the hydrolysis of dATP by βK211 which occurs twice as fast at pH 6.25 as at pH 8.0. On the basis of the ranking in Table 3.3, the substrate specificities of the mutant enzymes differ from the wild type. For example, the wild type enzyme hydrolyzes GTP faster than any other substrate shown at pH 8.0, but GTP is generally hydrolyzed slower than the other substrates by the mutant enzymes at either pH. UTP is hydrolyzed by the mutant enzymes faster than the other substrates at either pH, while the wild type enzyme hydrolyzes UTP slower than GTP, ITP, and dATP at pH 8.0. Furthermore, at a substrate concentration of 5.5 and a pH of 8.0 strains βN211, βD211, and βK211 hydrolyze UTP and ITP essentially as fast as ATP (ATP data not shown). Strain βA211 hydrolyzes UTP, ITP, and dATP faster than ATP at pH 8.0 (Fig. 3.3, Table 3.3). The enzyme from wild type yeast hydrolyzes ATP far faster than any of the alternative substrates shown.

Table 3.3 Hydrolysis of various nucleotide substrates by submitochondrial particles from mutant and wild type yeasts.

pH 8.0

SEY 103	GTP	ITP	dATP	UTP	CTP	XTP
	1.49	0.93	0.83	0.71	0.19	0.14
βN211	UTP	ITP	CTP	dATP	GTP	XTP
	0.24	0.20	0.13	0.10	0.08	0.05
βD211	UTP	ITP	dATP	CTP	GTP	XTP
	0.21	0.18	0.09	0.07	0.06	0.05
βK211	UTP	ITP	dATP	CTP	XTP	GTP
	0.18	0.15	0.07	0.06	0.03	0.03
βA211	UTP	ITP	dATP	CTP	XTP	GTP
	0.16	0.13	0.06	0.02	0.01	0.00

pH 6.25

SEY 103	ITP	GTP	UTP	dATP	XTP	CTP
	0.68	0.66	0.52	0.39	0.19	0.19
βN211	UTP	ITP	CTP	dATP	GTP	XTP
	0.19	0.16	0.13	0.10	0.08	0.05
βD211	UTP	ITP	CTP	dATP	XTP	GTP
	0.18	0.16	0.12	0.09	O.O5	0.05
βK211	UTP	dATP	ITP	CTP	XTP	GTP
	0.17	0.14	0.12	0.07	0.03	0.03
βA211	UTP	ITP	XTP	dATP	CTP	GTP
	0.13	0.10	0.02	0.02	0.00	0.00

Reaction velocities were determined at pH 6.25 and 8.0 by coupled assay at a substrate concentration of 5.5 mM, and are expressed in μmol/min X mg. Substrates were GTP, ITP, dATP, UTP, CTP, and XTP. Substrate concentrations were determined by absorbance at 260 nm. Values shown are averages of four measurements. Substrates are ranked from left to right by decreasing rate of hydrolysis by each mutant at each pH.

Comparison of ATP and dATP hydrolyses by wild type yeast F_1.

In the course of the previous experiment it was noted that the wild type yeast F_1-ATPase hydrolyzed dATP at slower velocities than several other nucleotides. This seemed odd in light of the finding that ATP was hydrolyzed by wild type F_1 at a greater rate than any other nucleotide tested (at 5.5 mM substrate). The only structural difference between ATP and dATP is the lack of a 2' hydroxyl group on dATP. The possibility of a contribution by this group to the transition state binding energy was investigated. Fersht (1985) and coworkers (Wilkinson et al., 1983) have shown that the transition state binding energy of an enzyme-substrate complex may be measured from the specificity constant k_{cat}/K_m. This relationship can be used to determine the contribution of a substitutable functional group to the transition state binding energy. The ratio of k_{cat}/K_m for dATP to k_{cat}/K_m for ATP is proportional to the difference in transition state binding energy between the two substrates.

$$\Delta G = RT \ln [(k_{cat}/K_m)_{dATP}/(k_{cat}/K_m)_{ATP}] \quad \text{(eq. 3.1)}$$

Values of k_{cat} and K_m for the hydrolysis of ATP and dATP by purified yeast F_1-ATPase were determined (Table 3.4). Application of equation 3.1 to these data shows that ATP is bound more stably in the transition state than dATP by 0.91 kcal/mol. This difference in binding energy is distributed almost equally between the k_{cat} and K_m terms, suggesting that dATP is not as tightly bound as ATP and

Table 3.4 Kinetic Constants for ATP and dATP Hydrolysis by Pure Yeast F_1.

	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($X10^5$ s^{-1} M^{-1})
ATP	0.42 ± 0.04	409 ± 13	9.65 ± .73
dATP	0.97 ± 0.09	182 ± 6	1.87 ± .12

Values for K_m and V_{max} were determined as described in Materials and Methods. K_{cat} was calculated using a M_r of 380,000 for yeast F_1-ATPase.

that conformational changes necessary for dATP hydrolysis may require more energy than those needed for ATP hydrolysis.

Discussion

H211 is Indirectly Involved in Substrate Binding

The results in Table 3.1 show that the each of the mutations at position 211 results in an increase in K_m. It is therefore concluded that H211 has either a direct or an indirect role in substrate binding. It is noteworthy that of the position 211 mutations, H211N has the least effect on K_m and is also the only one which supports aerobic respiration. Of all of the amino acids substituted for histidine at position 211 asparagine is the best structural analogue. As discussed in Chapter 2, asparagine may also be able to form hydrogen bonds in a manner similar to histidine. For these reasons it is concluded that the most important characteristic of the amino acid at position 211 is its shape and/or its ability to form hydrogen bonds. In contrast to the position 211 mutations, L203F does not affect K_m. In chapter 2 it was shown that L203F suppresses the phenotypes of H211D. H211K, H211I, and H211A. This suppressive effect is also expressed in the K_m values of the double mutants. Each double mutant has a K_m which is equal to or greater than wild type, but less than that of H211N. Thus the intragenic suppression by L203F also applies to H211N, and appears to be related to the relief of a nucleotide binding defect. This is consistent with the idea discussed in Chapter 2 where L203F alters the β subunit conformation such that the influence of position 211 is reduced or eliminated. This would support the

argument for an indirect role for H211 in nucleotide binding. The question then becomes: what is this indirect role? One possibility would be that H211 contributes to the structure of the active site or nucleotide binding site without direct physical involvement in catalysis or substrate binding.

H211 is Required for Correct Conformation of the Active Site

The fact that each of the substitutions for H211 results in an enzyme with reduced stability shows that this residue is required for proper conformation of the F_1 complex. It is possible that the ionization state of position 211 is important in this role. In order to investigate this possibility the pH optima for ATPase activity of the mutant enzymes were determined. As shown in Fig. 3.3, the pH optimum of each position 211 mutant is in the range of 6.0-6.5. The wild type optimum is pH 8.0. The fact that each mutation results in a similar alteration of the pH optimum suggests that the ionization state of position 211 is not important to its stabilization role. However, structural changes caused by the mutations apparently affect the catalytic mechanism.

In order to evaluate the effect of the mutations on the catalytic mechanism, the pK_a values for the groups involved in catalysis were determined. Log V_{max}/K_m vs pH plots for the wild type and for mutants βD211, βK211, and βN211 show that pK_{a2} is substantially decreased as a result of the substitutions at position 211 (Fig. 3.4, Table 3.2). Substitutions of amino acids with a basic group, an acidic group, or a non-ionizable sidechain for H211 affect the pK_{a2} value

similarly. This suggests that, in addition to stabilizing the structure of the complex, H211 is required for maintenance of proper active site conformation. This role is probably independent of the ionization state of histidine. It also appears that the loss of the histidine at position 211 can be compensated by a decrease in the solvent pH from the wild type optimum of 8.0 to a value of 6.0-6.5, again suggesting a structural rather than catalytic role for this residue.

The decrease in pK_{a2} values displayed by the mutant enzymes could owe to a microenvironmental effect on the group responsible for this pK_a. For example, the group in question could be a protonated lysine ε amino group with a pK_a of around 10.0. If the structure of the active site is altered such that this amino group is forced into a more hydrophobic environment, then it would tend to deprotonate at a lower pH. Alternatively, the the shift in pK_a may be due to a change in the identity of the group acting as base, implying a new catalytic mechanism. Both of these suggestions are consistent with a structural perturbation induced by substitution at position 211. The first explanation is simpler for the following reasons. Both positive catalytic cooperativity and negative cooperativity of substrate binding are still displayed by the mutant enzymes. In the case of βN211, the negative cooperativity is relieved by bicarbonate. These are phenomena which require the complicated interaction of several subunits. It seems unlikely that a conformational change which results in a new catalytic mechanism would preserve these

complex interactions. Furthermore, the data show that the pK_{a2} values of the mutant enzymes are in the range of 8.0-8.4 (Table 3.2). There are no naturally ocurring amino acid sidechain functional groups with a pK_a in this range, therefore an environmental effect on the true pK_a of the group in question is likely. This is the case whether a new mechanism is invoked or not. Therefore it is simpler to propose that the original catalytic mechanism is operating, and that the pK_a of the basic group has been altered as a result of a structural change in the active site caused by a substitution for H211.

Ionization Induced Structural Changes in the Mutant ATPases do not Restore Wild type Conformation

As shown in Fig. 3.2, the pH optimum for ATPase activity of each enzyme carrying a substitution at position 211 is 1.5 to 2 units less than the wild type optimum. One explanation for these shifts in pH optimum is that each of the mutations at position 211 causes a conformational change which is compensated at lower pH. This compensation could be the result of ionization-induced folding changes which partially restore wild type conformation. Fig. 3.5 shows that as pH decreases from 8.5 to 5.5, so do the differences between the transition state binding energies of the mutant and wild type enzyme:substrate complexes. This is consistent with a restoration of wild type conformation. However, if wild type conformation is restored, one would expect the substrate specificities of the mutant enzymes at pH 6.25 to resemble the wild type at pH

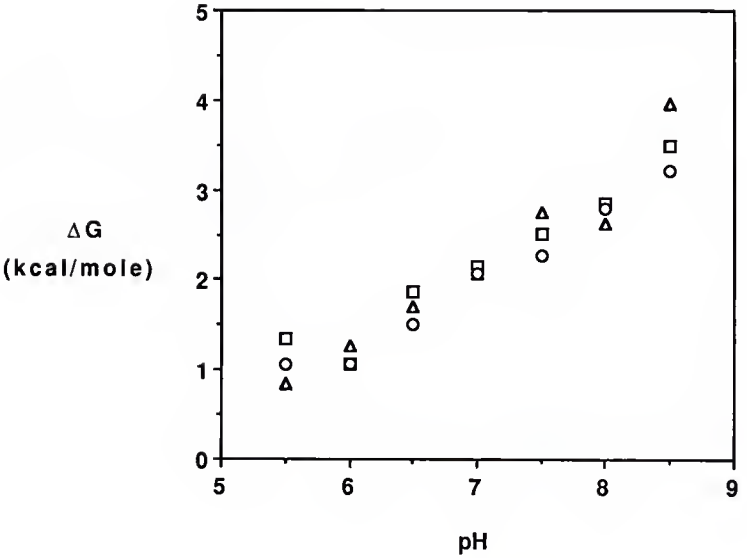

Fig. 3.5 The influence of pH on the difference in transition state binding energy between mutant ATPases and the wild type. Equation 3.1 was used to calculate a value for the difference (ΔG) in transition state binding energies between the wild type and mutant ATPases at each of 7 pH values. Because the actual concentrations of enzyme in each submitochondrial particle preparation are unknown, the abscissa scale is arbitrary. Comparisons of absolute value between data sets are inappropriate, however the trends are significant.

8.0. The ranking of substrates by hydrolysis rates (Table 3.3) suggests that the substrate specificities of the mutant enzymes are generally insensitive to the change in pH employed in the experiment. The idea of an ionization-dependent restoration of wild type conformation thus appears unlikely. A better explanation is that the mutant enzymes undergo ionization-induced structural changes which increase binding specificity for ATP without causing the enzyme to assume a more wild type conformation.

The 2' Hydroxyl Group of ATP Facilitates Binding and Catalysis

The contribution of a substrate functional group to the binding energy of the enzyme-substrate transition state can be determined by measurements of V_{max} and K_m (eq. 3.1 above, Wilkinson et al., 1983, Fersht, 1985). If these constants are determined for a series of enzyme substrates which differ only by the identity of this functional group, then the differences in binding energies of the various substrates can be determined. The contribution of a given functional group to the binding energy of the transition state can then be inferred. This type of experiment presupposes that the functional group in question is known to be involved in binding. If the group in question is not involved in binding, then differences in the calculated binding energy of the transition state actually reflect the energy required for enzyme conformational changes involved in accomodating the altered substrate in the active site.

V_{max} and K_m for hydrolysis of ATP and dATP by pure, wild type F_1 were measured, and the the binding energy of ATP in the

transition state was found to be more stable than dATP by 0.91 kcal per mole. This is in the range of energy represented by a hydrogen bond, so it is tempting to speculate that the role of the 2'OH of ATP is to participate in substrate binding through hydrogen bond formation. Although this type of experiment cannot show that the 2' hydroxyl group of ATP is bound by the enzyme in the transition state, the data do show that this group contributes to the geometry of the substrate in a way that facilitates binding and catalysis by the enzyme.

CHAPTER 4

ANALYSES OF RECOMBINANT REGULATORY SUBUNITS FROM BEEF HEART AND *E. coli* F_1-ATPASES

Introduction

The ε subunit of the *E. coli* F_1-ATPase (EF_1) is a 15 kd protein which has been shown to inhibit ATPase activity of soluble EF_1 noncompetitively with a K_i in the nM range (Sterneweis and Smith, 1977, Sternweis and Smith, 1980, Dunn et al., 1982). Exogenously added ε does not inhibit F_0F_1, but an increase in F_0F_1 ATPase activity has been correlated with trypsin cleavage of the endogenous ε subunit (Mendel-Hartvig and Capaldi, 1991). The ε subunit therefore seems to serve a regulatory function in the control of ATP hydrolysis. Such a function would be important at times when the cellular pool of ATP is depleted or at other times when the need for ATP synthesis is greater than that for ATP hydrolysis.

Mitochondrial F_1-ATPase inhibitor (F_1I) proteins have been discovered in a variety of organisms including animals (Pullman and Monroy, 1963), plants (Nelson et al., 1972), and fungi (Klein et al., 1977, Hashimoto et al., 1981). These proteins tend to be small (about 10,000 M_r), highly charged, and largely α helical. Beef heart F_1I is a noncompetitive inhibitor with a K_i in the μM range and it inhibits both the soluble and membrane-bound forms of the enzyme. Inhibition by F_1I is maximal at slightly acidic pH, and decreases as

pH becomes basic. The physiological role of F_1I is probably similar to that proposed for the EF_1 ε subunit, namely inhibition of ATP hydrolysis in times when ATP synthesis is needed. Furthermore, there is limited sequence homology between residues 55-65 of the ε subunit and residues 35-45 of F_1I (Fig 4.1). This region is also conserved in inhibitor proteins from *S. cerevisiae* and *C. utilis.* Synthetic peptides homologous to this segment of F_1I have been shown to inhibit beef heart F_1 in a pH dependent manner (Stoudt et al., 1992).

In this chapter, experiments have been performed which compare recombinant beef heart F_1I with recombinant EF_1 ε. The K_i values, pH dependence of inhibition, and cross-reactivity of F_1I with EF_1 have been evaluated. In addition, alanine scanning mutagenesis was used to identify important amino acid residues of F_1I in the segment from R35 to L45. The results show that regardless of any sequence homology, these two proteins are not likely to have the same mechanism of action.

Materials and Methods

Strains and Plasmids

E. coli K-12 strain BL-21(DE3) (F^-, *ompT*, $r_B^-m_B^-$) was used for the expression of recombinant F_1I. Plasmid $pALF_1I$-6 (FIG 4.2) was constructed by Van Heeke et al. for the expression of recombinant F_1I.

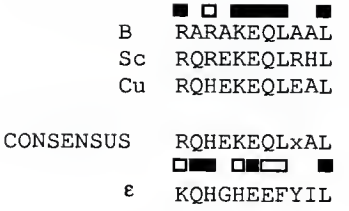

Figure 4.1 Homology between F_1-ATPase inhibitor proteins and the E. coli ε subunit. B: beef heart F_1I residues 35-45, Sc: *Saccharomyces cerevisae* F_1I residues 30-40, Cu: *Candida utilis* F_1I residues 30-40, ε: *E.coli* F_1-ATPase ε subunit residues 55-65. Filled boxes represent regions of sequence identity, open boxes represent regions of sequence homology.

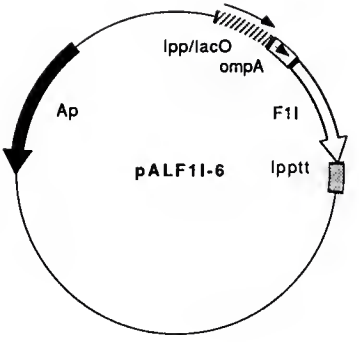

Figure 4.2 *E. coli* expression plasmid pALF$_1$I-6. This plasmid is derived from pINomp IIIa (Ghrayeb et al., 1984), and is modified to allow expression of native F$_1$I. Further details are given in Results. Abbreviations are Ap: ampicillin resistance gene (β lactamase), lpp: lipoprotein promoter, lacO: *lac* operator, omp A: outer membrane protein A signal sequence, F$_1$I: F$_1$ inhibitor protein gene, lpptt: lipoprotein transcription terminator.

Preparation of *E. coli* F_1-ATPase

F_1-ATPase was purified by the method of Futai et al. (1974) as modified by Smith and Sternweis (1977). Twenty-eight g (wet weight) of *E. coli* NM522 cells were collected from an overnight culture by centrifugation. The pellet was resuspended in 40 ml of 50 mM Tris (pH 8.0), 10 mM $MgCl_2$. Cells were lysed by two passes through a French Press (16000 lbs. pressure) and cell debris was removed by centrifugation at 8000 x g for 10 minutes at 4°C. Membranes were isolated from the supernatant fraction by centrifugation at 100000 x g for 90 minutes at 4°C, washed in 50 ml of 10 mM Tris-SO_4 (pH 7.5), 10 mM EDTA, 10% glycerol, recollected by centrifugation at 100000 x g, and stored at -75°C. F_1 was released from the membranes by resuspension in 20 ml of 1 mM Tris (pH 8.0), 0.5 mM EDTA, 10% glycerol and incubation for 60 minutes at room temperature. Membranes were removed by centrifugation, and the supernatant fraction was adjusted to 1 mM ATP and loaded onto a 10 x 2.5 cm column of Whatman DE-52 anion exchange resin equilibrated in 50 mM Tris (pH 8.0), 2 mM EDTA, 10% glycerol, 1 mM ATP, 1 mM dithiothreitol, 1 mM p-aminobenzamidine. The column was washed with 50 ml of equilibration buffer and F_1 was eluted with 200 ml of a linear gradient of 100 mM to 550 mM Tris buffer that was otherwise identical to the equilibration buffer. Fractions of 5-6 ml were collected. The twelve most active fractions were pooled, ammonium sulfate was added to 65% of saturation, and protein was allowed to precipitate overnight at 4° C. The precipitate was

collected by centrifugation at 12000 x g for 10 minutes at 4° C, and resuspended in 0.7 ml of 0.25 M sucrose, 15 mM Tris (pH 8.0), 1 mM EDTA, 1 mM p-aminobenzamidine. Ammonium sulfate was added to 32.5% of saturation and the suspension was stored at 4° C. The specific activity of this preparation, which was only partially depleted of the ε subunit, was 14.21 $\pm$ 0.45 μmol ATP $min.^{-1}$ mg^{-1} F_1.

Preparation of Beef Heart F_1-ATPase

Beef heart F_1-ATPase, depleted of F_1I, was purified from mitochondrial paste by the method of Knowles and Penefsky 1972a, 1972b) as modified by Gruys et al. (1985). Mitochondrial paste was thawed and diluted to 3 mg protein/ml in 0.25 M sucrose, 10 mM Tris, 1 mM PMSF, 1 mM PABA, pH 7.5. Aliquots of 90 ml were sonicated for 3 minutes in an ice water bath during which time the temperature of the particles did not rise above 28° C. The suspension was cleared by centrifugation for 10 minutes at 12000 X g and 4° C. Submitochondrial particles were collected by by centrifugation for 90 minutes at 100000 X g and 4° C, and resuspended in 50 ml of solution B (0.1 M sucrose, 4 mM ATP, 2 mM EDTA, 1 mM PMSF, 1 mM PABA). The protein concentration of this suspension was 32 mg/ml and the pH was 7.1. The pH was then adjusted to 9.2 by addition of NH_4OH and the suspension was incubated overnight at room temperature to allow the dissocation of F_1I. The pH was then readjusted to 9.2 and submitochondrial particles depleted of F_1I were collected by centrifugation at 100000 x g for 90 minutes. The pellet was resuspended in 150 ml of solution

B (protein concentration was 1.4 mg/ml), pH was adjusted to 9.2 with NH_4OH, and F_1 was released by sonication for a total of 12 minutes with cooling in a room temperature water bath. Debris was removed by centrifugation at 100000 X g for 90 minutes. The suspension was cleared further by addition of 3 N glacial acetic acid to pH 5.4. Precipitated proteins were removed by centrifugation at 26000 X g for 5 minutes and the pH of the supernatant was quickly adjusted to 8.0 by addition of 2 M unneutralized Trizma base. This solution was loaded onto a 4 X 15 cm column of DEAE-50 Sephadex equilibrated with solution C (20 mM Tris, 4 mM ATP, 2 mM EDTA, 1 mM PMSF, 1 mM PABA). The column was washed 150 ml of solution C and then with 175 ml of solution D (100 mM Na_2SO_4, 20 mM Tris, 4 mM ATP, 2 mM EDTA, 1 mM PMSF, 1 mM PABA). F_1 was eluted with solution E (solution D with 150 mM Na_2SO_4). Fifteen fractions of 9 ml were collected and assayed for ATPase activity. Fractions 6-11, containing 81% of the activity, were pooled and solid $(NH_4)_2SO_4$ was added to 82% of saturation. The suspension was incubated overnight at 4° C, and the precipitate was collected by centrifugation at 13,000 X g for 10 minutes at 4° C. The precipitate was resuspended in 0.6 ml of 0.25 M sucrose, 50 mM Tris, 4 mM ATP, 1 mM PMSF, 1 mM PABA, and solid $(NH_4)_2SO_4$ was added to 60% of saturation. A total of 3.6 mg of F_1 was recovered. The specific activity of this preparation was 42 μmoles $min.^{-1}$ mg^{-1}.

Purification of F_1I

E. coli BL21(DE3) carrying pALF$_1$I-6 were grown with shaking in 10 ml of LB with 1 mg ampicillin overnight at 37° C. An aliquot of this culture was diluted 1:100 in 250 ml, 500 ml, or 1 L of fresh L-broth with ampicillin and grown at 37° C to an optical density at 550 nm of 0.8-1.0. Expression of F_1I was then induced by addition of IPTG to 2 mM and subsequent growth for 3 to 5 hours at 37° C. Cells were collected by centrifugation and frozen at -75° C for at least 30 minutes. The cells were then thawed at room temperature, osmotically shocked by resuspension in 1/10 culture volume of ice cold 1 M Tris, 2 mM EDTA pH 9.0, and incubated for 30 minutes on ice. All subsequent manipulations were performed at 0-4° C. The periplasmic fraction was cleared by centrifugation at 3000 X g, and dialyzed against 3 changes of 100 volumes of Buffer A (20 mM Na_2PO_4, 0.5 mM EDTA, 0.5 mM EGTA, pH 6.7). The dialyzed periplasmic fraction was loaded onto a Cellex CM cation exchange column which was equilibrated in buffer A. The column was washed with 30 ml of buffer A at a flow rate of 0.5 ml/minute. F_1I was eluted at a flow rate of 0.5 ml/minute with 60 ml of a pH and ionic strength gradient starting with buffer A and ending with 100 mM Na_2PO_4, 0.5 mM EDTA, 0.5 mM EGTA, pH 8.7. Fractions of 5.0 ml were collected and 20 µl aliquots of each fraction were assayed by SDS-PAGE on 10% Tris-Tricine gels (Schagger and von Jagow, 1987). The fractions containing pure F_1I were pooled, concentrated to less than 2 ml with Amicon centriprep 10 centrifugal concentrators,

dialyzed against 3 changes of 1000 volumes of distilled, deionized H_2O and stored at -20° C.

The protein concentration of a sample of pure wild type recombinant F_1I was determined by amino acid analysis performed by the Protein Chemistry Core Facility of the University of Florida Center for Biotechnology Research. A portion of the same sample was then used to construct a standard curve by means of the bicinechoninic acid copper chelation assay (BCA assay, Pierce). The protein concentration of all subsequent preparations of F_1I was determined by the BCA assay and reference to the standard curve. N-terminal peptide sequencing was performed by the Protein Chemistry Core Facility. Site-directed mutagenesis and sequencing of the mutated clones was performed in the laboratory of Gino Van Heeke.

Assays of Activity

The ε subunit of *E. coli* F_1-ATPase was prepared by Judy Couton in the laboratory of Gino Van Heeke. To assay the effect of added ε subunit (or added hCA:ε fusion protein) on ATP hydrolysis by EF_1, ATPase activity was measured at a fixed substrate concentration in the presence of ε subunit concentrations ranging from 0.25 mole ε/mole EF_1 to 25 mole ε/mole EF_1. ATP hydrolysis was measured at 30°C by a coupled assay in an ATP regenerating system using pyruvate kinase and lactic dehydrogenase (Ebel an Lardy, 1975). The system consisted of 20 mM Tricine (pH 8.0), 0.45 mM NADH, 2 mM phosphoenolpyruvate, 6.5 mM KCl, 2 mM $MgCl_2$ and 2 units/ml

of both lactate dehydrogenase and pyruvate kinase. MgATP was added to 0.4 mM, this substrate concentration allowed the assay to be carried out for at least 10 minutes at an enzyme concentration of 2 μg/ml. Reactions were started by adding MgATP, purified ε or hCA:ε, and then EF_1 to the assay mixture. ATP hydrolysis was assayed for 10 minutes and rates were calculated from 4 to 10 minutes of assay.

The effect of added ε subunit on EF_1 V_{max} and K_m was determined by analysis of V vs [S] plots. Rates of ATP hydrolysis by EF_1 were measured at 30°C by coupled assay at 10 different substrate concentrations from 33.5 μM to 6 mM in the presence or absence of a 10-fold molar excess of added ε subunit. Reactions were started by addition of enzyme without preincubation of EF_1 and ε, and rates were calculated from the linear range of the reactions. The Enz-Fitter program was used to fit the data to the Michaelis-Menten equation and to calculate K_m and V_{max}.

ATP hydrolysis by BF_1 was measured at 30° C and pH 6.7 by the coupled assay described above. Inhibition by F_1I was assayed by addition of various amounts of F_1I to the reaction mixture followed by the addition of beef heart F_1 to begin the reaction. Rates were calculated after 2 minutes of assay in order to allow for slow, turnover-dependent conformational changes required for inhibition by F_1I (Panchenko and Vinogradov, 1985).

Results

Functional Tests of Recombinant F_1I and E. coli ε

The design, construction and optimization of expression of a synthetic gene encoding beef heart F_1I was performed in the laboratory of Gino Van Heeke (Van Heeke et al., in preparation). A system was also developed for the expression and purification of the EF_1 ε subunit (Van Heeke et al., submitted). The ε subunit was expressed as a fusion to the C-terminus of human carbonic anhydrase II (hCA), purified by affinity chromatography, and cleaved from hCA by enterokinase to yield authentic ε. Cleaved hCA was removed by affinity chromatography yielding pure ε. The expression and purification of ε was performed by Judy Couton and Regina Shaw. F_1I was expressed with the outer membrane protein A (omp A) signal sequence at its N-terminus. This resulted in secretion of F_1I into the periplasmic space where the signal sequence was cleaved. F_1I was then purified by cation exchange chromatography as described in Methods. Correct cleavage of the ompA signal sequence, which results in the mature form of F_1I, was confirmed by N-terminal peptide sequencing of the purified protein. Purified ε, hCA:ε fusion protein, and F_1I were then assayed for activity.

The activity of the purified recombinant ε subunit was assayed by determining its effect on K_m and V_{max} for ATP hydrolysis by EF_1. The preparation of EF_1 used in these measurements was only partially depleted of endogenous ε subunit. In the absence of added ε subunit, values for K_m and V_{max} were determined to be 0.092 $\pm$

0.015 mM and 14.21 ± 0.45 μmol ATP min^{-1} mg^{-1} EF_1 respectively. In the presence of a 10-fold molar excess of ε these values were 0.097 ± 0.014 mM and 7.89 ± 0.23 μmol ATP min^{-1} mg^{-1} EF_1. This decrease in the value of V_{max} with relatively little change in K_m is consistent with the previous characterization of ε as a noncompetitive inhibitor of EF_1 (Sternweis and Smith, 1980).

K_i values were estimated for both the ε subunit and the hCA:ε fusion protein. The rate of ATP hydrolysis by EF_1 was measured at a substrate concentration of 0.4 mM and pH 8.0 with varying amounts of added ε subunit or hCA:ε fusion protein. The ratio of ε or hCA:ε to EF_1 was varied from 0.25 to 25 moles per mole EF_1. The concentration of EF_1 in the assays was 5 nM. Figure 4.3 shows the effects of added ε or hCA:ε on ATP hydrolysis. The presence of the hCA fusion protein appears to make little difference in the inhibition pattern of ε. Half maximal inhibition occurs at an ε or hCA:ε concentration of about 10 nM, and plots of 1/V vs [I] yield K_i values of 11 nM for both ε and hCA:ε (Fig 4.4). These values agree well with previously published estimates of K_i for inhibition of partially ε-depleted EF_1 by added ε (Smith Sternweis, 1977, Sternweis and Smith, 1980). This experiment suggests that the N-terminus of the ε subunit is not required for interaction with the other F_1 subunits. Dallman et al. (1992) show that a zero-length crosslink can be formed between ε and β. This crosslink occurs between amino acid residues on peptide fragment D380-M431 of β and E96-M138 of ε. The data in this chapter suggest that ε folds such that the E96-M138

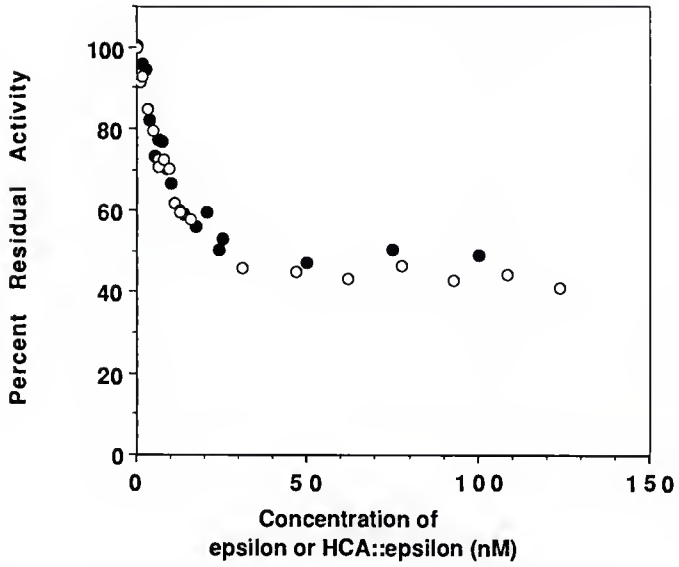

Fig. 4.3 Inhibition of *E. coli* F_1-ATPase by purified recombinant ε and hCAII:ε. ATP hydrolysis was measured as described in Materials and Methods in the presence of various amounts of added ε subunit or hCA:ε fusion protein. Reactions were started by the addition of enzyme and rates were calculated from 4 minutes to 10 minutes of assay. Rates are expressed as the percent of the rate observed in the absence of added ε or hCA:ε. The concentrations of MgATP and F_1-ATPase were 0.4 mM and 5 nM respectively. Closed circles represent recombinant ε and open circles represent hCA:ε fusion protein.

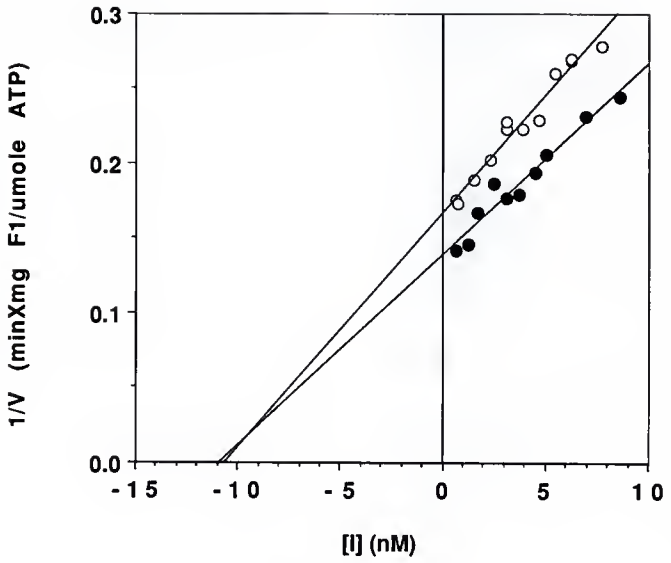

Fig. 4.4 Dixon plot of inhibition of *E. coli* F_1-ATPase by ε and hCA:ε. Initial velocities of ATP hydrolysis were measured as described in Materials and Methods. The concentrations of MgATP and *E. coli* F_1-ATPase were 0.4 mM and 5 nM respectively. Closed circles represent recombinant ε, and open circles represent hCA:ε fusion protein.

segment is not near to the N-terminus. The ε subunit has also been crosslinked to the γ subunit (Bragg and Hou, 1980), but the linkage has not been peptide mapped.

The biological activity of recombinant F_1I was evaluated by measuring the initial velocity of ATP hydrolysis by beef heart F_1-ATPase at 0.4 mM ATP and pH 6.7 while varying the concentration of F_1I. Inhibition of F_1-ATPase activity by authentic F_1I is slow and requires many turnovers unless the inhibitor is added to the reaction at pH 6.7 (Cintron et al., 1982). Recombinant F1I was added to reaction cocktails at pH 8.0 and turnover dependent inhibition was observed (Table 4.1). Maximal inhibition was found to occur at between 1.6 and 2 μM F_1I, with half maximal inhibition at about 0.5 μM (Fig. 4.5). A K_i value of 0.1-0.2 μM was determined by plotting 1/V vs [F_1I] at 5 different ATP concentrations (Fig. 4.6). The characteristics of inhibition by F_1I over a broader range of concentration and at varying ionic strengths were also assessed. Inhibition by F_1I at concentrations up to 10 μM and at ionic strengths of 19 mM, 124 mM and 176 mM was measured. The ionic strength of the standard assay buffer was 19 mM and potassium chloride was added to reach ionic strengths of 124 mM and 176 mM. Initial velocities of ATP hydrolysis by beef heart F_1 were measured at pH 6.7 and 0.4 mM ATP. The results are shown in Fig. 4.7. Inhibition of beef heart F_1 activity reached a maximum at an F_1I concentration of 0.8 to 2 μM and decreased at greater concentrations. The rate of ATP

Table 4.1 Turnover dependence of recombinant F_1I.

	RATES (μmol/min.mg)	
[bmF_1I] (μM)	0 - 1 min	6 - 9 min
0.00	25.6	22.7
0.16	22.8	17.7
0.40	23.1	15.5
0.80	19.9	8.7
1.60	22.9	6.6

Beef heart F_1-ATPase activity was measured at pH 6.7 in the presence of various concentrations of purified F_1I as described in Material and Methods. The inhibitor protein was added from a 41 μM stock solution at pH 8.0. The data represent a single experiment which was reproduced twice. Absorbance at 340 nm was recorded every 13 seconds during the experiment.

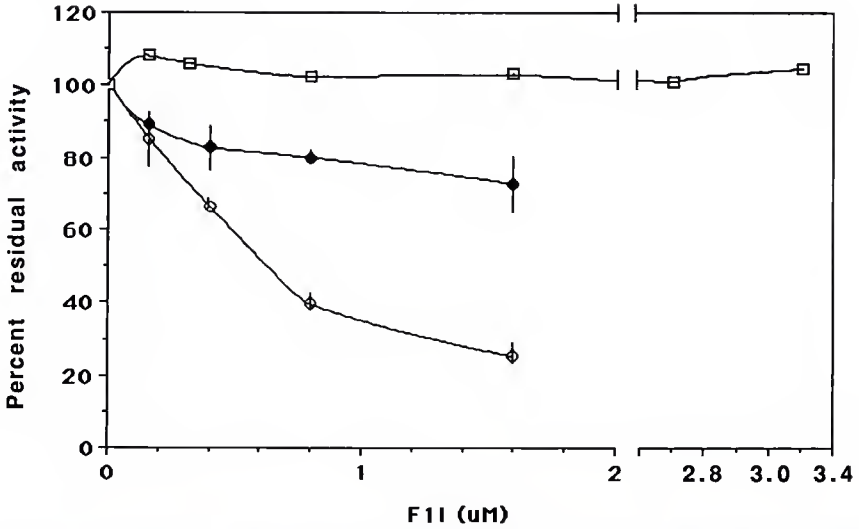

Fig. 4.5 Inhibition of beef heart and *E. coli* F_1-ATPases by recombinant F_1I. F_1-ATPase activity was assayed as described in Materials and Methods. Activities are expressed as a percentage of the rates measured in the absence of F_1I. Open circles - beef heart F_1 pH 6.7, closed circles - beef heart F_1 pH 8.0, Rates are averages of 3 measurments, error bars show range of data. Open squares - *E. coli* F_1 pH 6.7, rates are single measurments. Reactions were carried out for 10 minutes and rates were calculated from 6-9 minutes to allow slow, pH dependent conformational changes of F_1I to occur. Substrate concentrations were 0.4 mM at pH 6.7 and 0.3 mM at pH 8.0.

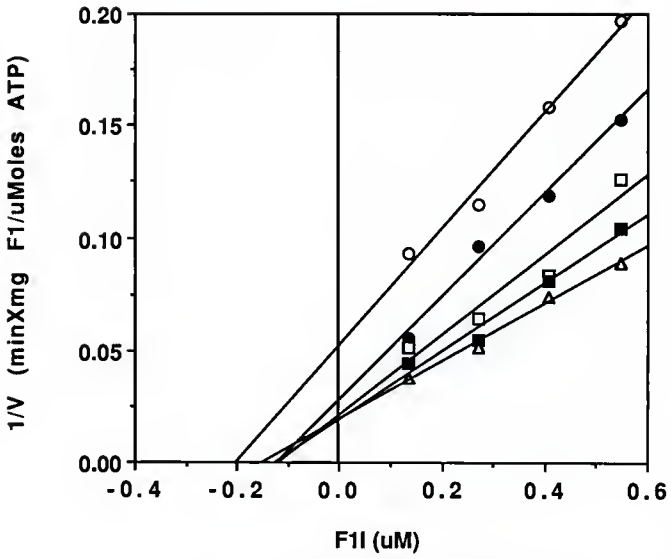

Fig. 4.6 Dixon plot of inhibition of beef heart F_1-ATPase by F_1I. Initial velocities of ATP hydrolysis were measured as described in Materials and Methods. ATP concentration was varied as follows, open circles: 0.6 mM, closed circles: 0.25 mM, open squares: 0.75 mM, closed squares: 1.5 mM, triangles: 3 mM.

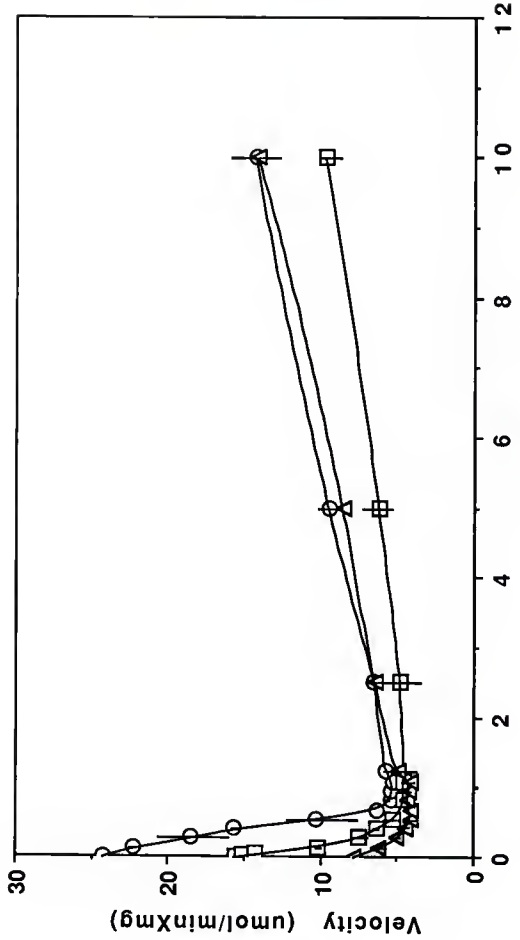

Figure 4.7 The effect of ionic strength on inhibition of beef heart ATPase activity by F_1I. Initial velocities of ATPase activity were measured as described in methods. Data points are either single measurements or are the averages of 2 to 5 measurements. Error bars indicate the range of the data.

hydrolysis was negatively affected by increasing ionic strength, but the profile of inhibition by F_1I was essentially unaffected.

Effect of pH on Activity.

F_1I has a characteristic pH dependence, it inhibits best at pH values around 6.7 but does not inhibit well at the pH optimum of the enzyme which is 8.0. This dependence was demonstrated for the recombinant enzyme by assaying ATPase activity in the presence of varying amounts of recombinant F_1I at either pH 6.7 or 8.0 (Fig. 4.5). Activity was inhibited by 80% at pH 6.7 but only by 20% at pH 8.0. This experiment was repeated for the recombinant ε subunit in order to see if it showed a similar pH dependence (Fig 4.8). It was found that the ε concentration required for half maximal inhibition was less at pH 6.7 than at pH 8.0, but the extent of inhibition was greater at pH 8.0. These results suggest that the mode of action of ε is different than that of F_1I.

Effect of F_1I on *E. coli* F_1

It has been shown that the EF_1 ε subunit does not inhibit mitochondrial ATPases (Nelson et al.,1972, Nieuwenhuis et al., 1974), this finding was reproduced here (data not shown). Mitochondrial F_1I does inhibit chloroplast ATPase (Hasebe et al, 1977) which is similar to EF_1 both structurally and in terms of its gene organization (Munn et al., 1991. The effect of F_1I on EF_1 was evaluated by measuring the activity of EF_1 at pH 6.7 with varying amounts of F_1I. As shown in Fig. 4.5, concentrations of F_1I up to 3.2 μM had no inhibitory effect on EF_1.

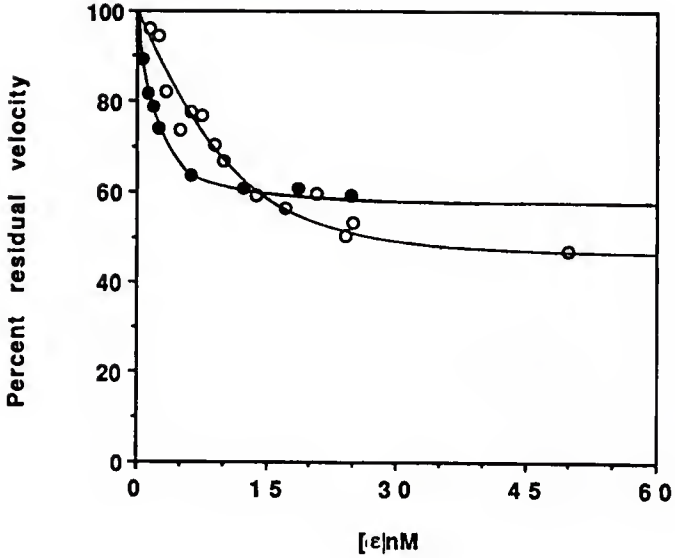

Fig. 4.8 The effect of pH on inhibition of *E. coli* F_1-ATPase activity by recombinant ε subunit. Initial velocity was measured as described in Materials and Methods. The concentrations of *E. coli* F_1-ATPase and MgATP were 5 nM and 4 nM, respectively. Activities are expressed as a percentage of the rate measured in the absence of added e subunit. Each data point represents a single measurement. Open circles: pH 8.0, closed circles pH 6.7.

Identification of Critical Amino Acid Residues of F_1I by Alanine Scanning Mutagenesis

Alanine scanning mutagenesis was performed in the region from R35 to L45 of F_1I. Seven different alanine substitution mutations were constructed as shown in Table 4.2. The wild type and mutated proteins were expressed and purified as described above. In order to determine K_i for inhibition of beef heart F_1 by the wild type recombinant F_1I, initial velocities for ATP hydrolysis were measured at each of five different substrate concentrations in the presence of each of five different concentrations of F_1I. Negative cooperativity of ATP binding was evident when the initial velocity data were plotted as 1/V vs 1/[ATP] (Fig. 4.9). The nonlinearity of these curves makes it difficult to determine K_i by replots of slope against $[F_1I]$. This problem was addressed by using ITP rather than ATP as substrate. Binding of ITP by beef heart F_1 is known to show less negative cooperativity than binding of ATP. A double reciprocal plot of ITP hydrolysis by BF_1 confirms this (Fig. 4.10). Initial velocities for ITP hydrolysis were measured at each of five different substrate concentrations in the presence of each of five different concentrations of F_1I. Plots of 1/V vs 1/[ITP] are linear at $[F_1I]$ less than 0.3 μM, but the curves become nonlinear at $[F_1I]$ greater than 0.3 μM (Fig. 4.11). Due to this result it was decided that values for K_i would be estimated from plots of 1/V vs [I] with ATP as substrate rather than from slope replots with ITP as substrate.

Table 4.2 The effects of alanine substitutions in the region from R35 to L45 of the beef heart F_1-ATPase inhibitor protein.

35 40 45	mutation	maximum inhibition (%)	K_i (μM)
R A R A K E Q L A A L	wildtype	80	0.1-0.2
A - - - - - - - - - -	R35A	46	1.0-1.7
- - A - - - - - - - -	R37A	65	0.7-1.3
- - - - A - - - - - -	K39A	87	0.1-0.3
- - - - - A - - - - -	E40A	45	0.6-0.85
- - - - - - A - - - -	Q41A	25	7.0-12.0
- - - - - - - A - - -	L42A	0	--------
- - - - - - - - - - A	L45A	56	1.5-4.0

Maximum inhibition was determined by measuring initial velocity at pH 6.7 and 0.25 mM MgATP for increasing concentrations of F_1I. Values for K_i were determined by Dixon plot as discussed in Results.

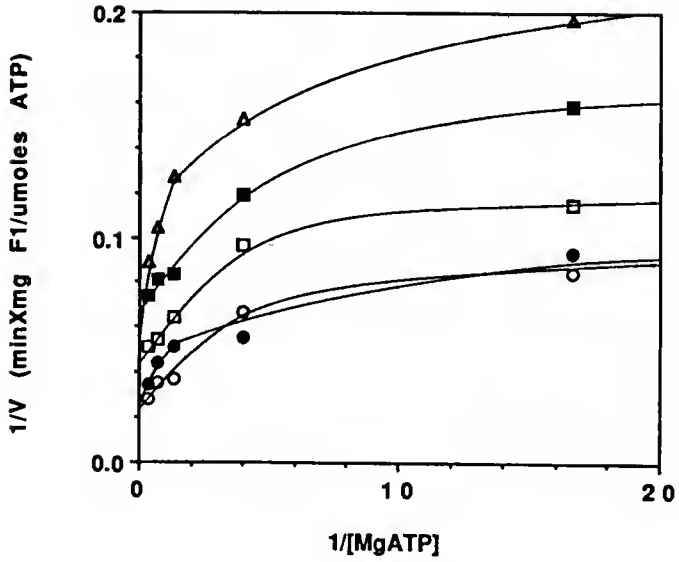

Fig. 4.9 Lineweaver-Burke plots of ATP hydrolysis by beef heart F_1-ATPase in the presence of varying amounts of F_1I. Initial velocities were measured as described in Materials and Methods at ATP concentrations of 0.06 mM, 0.25 mM, 0.75 mM, 1.5 mM, and 3 mM. Most data points are averages of two measurements, some represent single measurments. The concentration of beef heart F_1-ATPase in the assays was 2.6 nM. F1I concetrations were as follows, triangles: 0.546 μM, closed squares: 0.409 μM, open squares: 0.273 μM, closed circles: 0.136 μM, open circles: 0 μM.

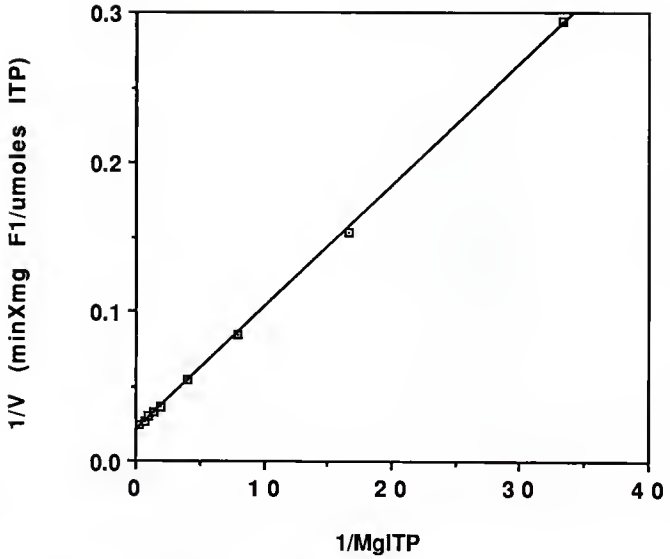

Fig. 4.10 Lineweaver-Burke plot of MgITP hydrolysis by beef heart F_1-ATPase. Initial velocities were measured as described in Materials and Methods. Enzyme concentration was 2.6 nM and MgITP was varied over a range of 0.03 mM to 3 mM. ITP concentration was determined by absorbance at 260 nm using a molar extinction coefficient of 1.44 X 10^4. In order to ensure that no IDP was present at the start of the reactions, it was necessary to incubate the reaction mixture before the addition of F_1-ATPase for 10 minutes at 30° C to allow the coupling system to regenerate ITP from IDP. Reactions were then started by addition of F_1.

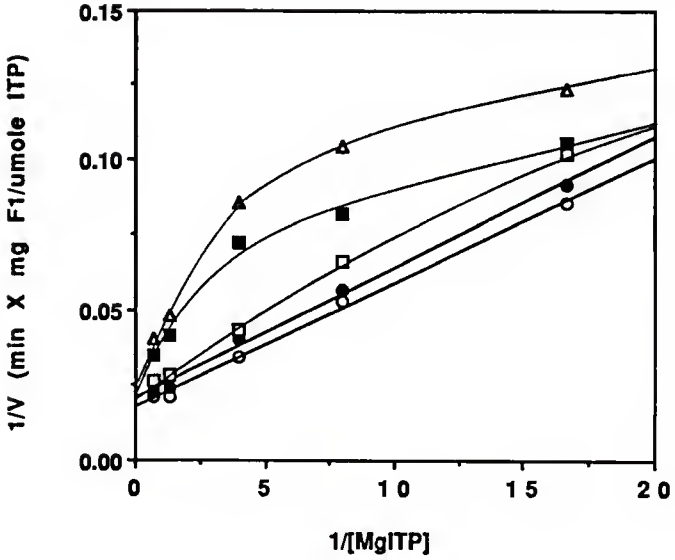

Fig. 4.11 Lineweaver-Burke plots of ITP hydrolysis by beef heart F_1-ATPase with varying amounts of F_1I. Initial velocities were measured as described in Materials and Methods and Fig. 4.10 at ITP concentrations of 0.06 mM, 0.125 mM, 0.25 mM, and 1.5 mM. Data points represent single measurements. Concentrations of F1I are as follows, triangles: 0.546 μM, closed squares: 0.409 μM, open squares: 0.273 μM, closed circles: 0.136 μM, open circles 0 μM.

Inhibition by the wild type and various mutant F_1I proteins was assayed at pH 6.7 using four or more different concentrations of inhibitor protein at each five different concentrations of ATP. Examples of plots of 1/V vs $[F_1I]$ are shown in Figs. 4.6, and 4.12. K_i values were estimated from these plots. Values for maximal inhibition were taken from titration curves of the inhibitor at a substrate concentration of 0.25 mM. The results are presented in Table 4.1. Mutant R39A shows the least affect of all the mutants with little change in K_i and a slightly greater maximum inhibition (87%) than wild type. Mutants R35A, R37A, E40A, and L45A inhibit ATPase activity by 45-65% and have K_i values ranging from 4-fold to 20-fold above wild type. Q41A inhibits by only 25% and has a K_i about two orders of magnitude greater than wild type. The L42A mutation results in a complete loss of inhibitory activity.

Discussion

EF_1 ε and Beef Heart F_1I Do Not Have Similar Mechanisms of Action

E. coli F_1 ε and recombinant beef heart F_1I were expressed in E. coli, purified, and shown to have their appropriate inhibitory activities. Stout et al. (1992) have shown that a peptide homologous to a short segment of F_1I inhibits mitochondrial ATPase in a pH dependent manner similar to the authentic inhibitor protein. The peptide includes a region of ten amino acids which are homologous to a short region of the EF_1 ε subunit. This raised the question of whether ε and F_1I might have similar mechanisms of inhibition. The results of this chapter suggest that while both of these proteins are

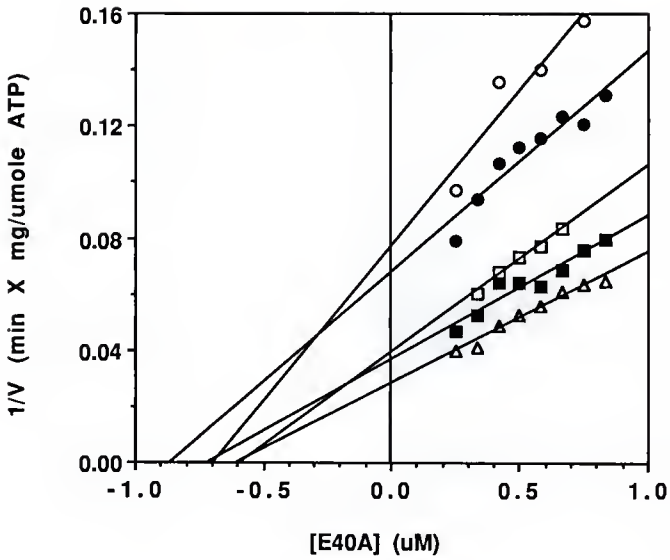

Fig. 4.12 Dixon plot of inhibition by F_1 inhibitor protein mutant E40A. Initial velocities were measured as described in Materials and Methods and Results at ATP concentrations of 0.075 mM (open circles), 0.15 mM (closed circles), 0.25 mM (open squares), 0.75 mM (closed squares), and 1.5 mM (triangles). Each data point represents the average of two measurements.

negative regulators of ATPase activity, they probably act in different ways. Unlike F_1I, the ε subunit functions about as well at pH 6.7 as it does at pH 8.0. Furthermore, F_1I has no inhibitory effect on EF_1, and EF_1 ε has no inhibitory effect on beef heart F_1. On the basis of these results it is concluded that the ε and F_1I proteins inhibit their respective ATPases through different mechanisms. An unexpected result which proved informative was the similarity in biological activities and K_i values of the purified ε protein and the hCA:ε fusion protein. The presence of a 30 kd N-terminal peptide apparently did not hamper the ability of the ε subunit to perform its function. This suggests that the N terminal sequences of ε are not necessary for interaction with EF_1, or for proper folding of the subunit. Codd et al. (1992) have recently isolated crystals of recombinant EF_1 ε which are of sufficient quality for X-ray crystallography. Their recombinant ε has two N-terminal substitution mutations, A1G and M2S, which do not affect the biological activity of the protein as shown by complementation tests. This is consistent with the current finding that the N-terminus of ε is not essential for activity.

Residues 55-65 of ε are similar in sequence to residues 35-45 of F_1I. The results of this study showed that these residues are important for inhibition of beef heart F_1 by F_1I. Kuki et al. (1988) have demonstrated that this region of ε is not sufficient for inhibition of EF_1. These authors used a series of ε subunit C-terminal deletion mutants to demonstrate that the residues between 73 and 80 are probably involved in binding F_1 to F_0. They also found that two

deletion mutants retaining only the N-terminal 80 and 93 amino acids respectively, both bound to purified F_1, and the 93 amino acid peptide was capable of inhibiting EF_1 whereas the 80 amino acid peptide was not. Thus the residues from 80-93 are essential for inhibition. These data support the conclusion reached here that EF_1 ε and F_1I are not equivalent and do not have the same mode of action.

Identification of Critical Amino Acid Residues in Beef Heart F_1I

The results of alanine scanning mutagenesis of residues 35-45 of F_1I show that some residues within this region are required for inhibition, while mutation of residues K39 and E40 has little or no effect. Circular dichroism spectra (Frangione et al., 1981, Van Heeke et al., 1993) and secondary structure prediction algorithms (Chou and Fasman, 1974) suggest that F_1I is highly α helical, and that the region from R35 to L45 is contained within an α helix. In Fig. 4.13 the region from R35 to I53 has been modeled on a helical wheel. The K_i values which result from the substitution of alanine for a given residue of F_1I are shown. The mutations resulting in the highest K_i values are clustered on one side of the helix. Harris (1984) has plotted the helical residues in the region from positions 33-50 with respect to hydrophobicity, and the resulting model shows that the helix is amphiphilic (shown in Fig 4.13). The two mutations with the least effect on K_i (K39A and E40A) occur in the predicted hydrophilic portion of the helix. All the other mutations, including L42A which eliminates inhibition, occur in the hydrophobic region of the helix.

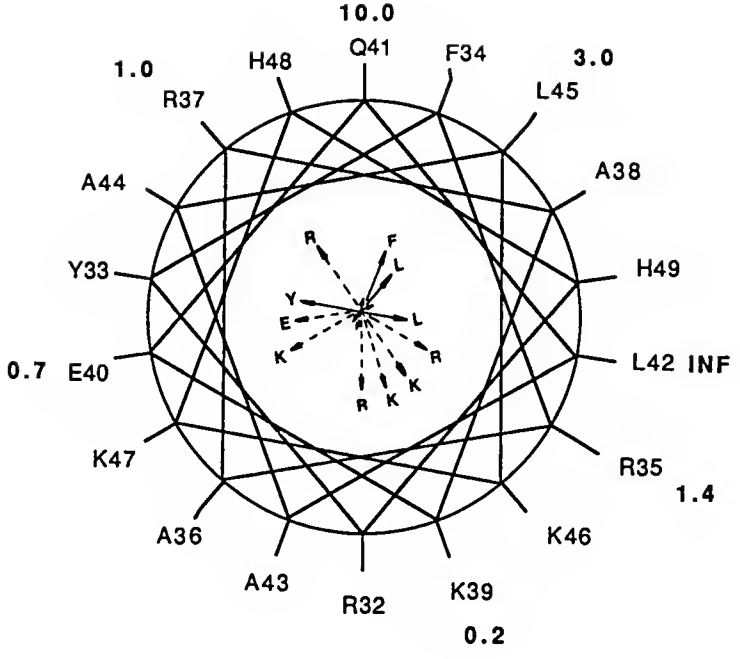

Fig. 4.13 Helical wheel arrangement of residues 32-49 of the beef heart F_1-ATPase inhibitor protein. Bold-face numbers are placed next to residues which were mutated to alanine, and represent the estimated Ki values of the resulting inhibitor proteins (units are μM, INF = infinite). Harris (1984) has presented a helical plot of residues 32-49 in which the hydrophobicity of each residue corresponds to the length of a vector. This plot is reproduced in the center of the helical wheel. Hydrophobicity is represented by solid lines and hydrophilicity is represented by broken lines.

One explanation of the data is that the hydrophobic face of the helix mediates interaction with the β subunit of F_1. Specifically, the substitution of alanine for certain residues may remove side chains which are involved in the hydrophobic interaction of F_1I and β. This has the result of interfering with inhibition by F_1I. It is unlikely that the substitutions of alanine for amino acids in this short region destabilize the α helix. Chou and Fasman (1974) show that alanine is one of the most frequently ocurring amino acid in α helices. It is more likely that the side chains removed by mutation are required for stable interactions with the β subunit.

Klein et al. (1982) have shown that F_1I forms oligomers in solution. Van Heeke et al. (1993) have noted that the C-terminus of F_1I contains hydrophobic residues arranged in a heptad repeat, and have analyzed the potential for F_1I to form a coiled coil structure. which might explain the oligmerization phenomenon. A computer program was written by Dr. Gerry Shaw which evaluates the potential for coiled coil formation. The program examines a peptide sequence in each of seven possible heptad repeat "reading frames". These reading frames are compared to those of known coiled coil proteins. For each amino acid in a given heptad, a score is calculated which reflects the frequency with which that amino acid occurs in a known coiled coil. The geometric mean of the scores is then calculated for windows of 28 amino acids. The resultant is compared to those calculated for known coiled coils. The scores calculated for F_1I showed that it is very likely that it forms a coiled coil, the scores

in some cases being higher than those for known coiled coil proteins. If F_1I forms a coiled coil structure, then this structure may be involved in the mechanism of inhibition. It has been reported (Klein et al., 1980) that the stoichiometry of binding of F_1I to F_1 is 1:1. Their data show binding stoichiometries of 1.0-1.6 moles of F_1I per mole of F_1. If this stoichiometry estimate is slightly low, then it is conceivable that F_1I binds to F_1 as a coiled coil dimer, and that coiled coil formation is necessary for inhibition. In this context the effect of the alanine scanning mutations might be to interfere with the hydrophobic interactions required for dimerization. Alternatively, dimerization may be a mechanism of F_1I sequestration. In this case, the effect of the mutations would probably be to interfere with F_1I-β subunit hydrophobic interactions as suggested above. The precise nature of the defects caused by the alanine substitutions is not at all clear. It is difficult to predict the effect of a substitution of an alanine for another hydrophobic amino acid in the hydrophobic face of an amphiphilic helix. It seems unlikely that a single such mutation would seriously interfere with the hydrophobic interactions required for a coiled coil structure or with nonspecific hydrophobic interactions in general.. It is more likely that the amino acid residues which were intolerant of alanine substitution are critical for the tertiary structure of F_1I or are required for specific structural interactions between F_1I and the β subunit which may be hydrophobic in nature.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Duncan et al. (1986) have proposed a three dimensional model for the nucleotide binding site of the F_1-ATPase β subunit which is based upon the high resolution NMR structure of adenylate kinase (Fig. 2.3). In this model, the adenine moiety of ATP interacts with a hydrophobic cleft which is composed of an α helix, a β-turn, and a β strand (Figs 2.1-2.3). Garboczi et al. (1988) have examined the function of this region through the use of synthetic peptides, and have proposed that a conserved histidine residue in this region is involved in nucleotide binding through charge interaction. The role of the homologous histidine of adenylate kinase (H36) has been the source of controversy for several years. Initially this residue was thought to play a role in the catalytic mechanism (Tian et al., 1988), but later studies (Fry et al., 1985, 1987) supported a role only in nucleotide binding. The most recent work reports the use of site-directed mutagenesis to probe the role of H36 (Tian et al., 1988). Substitution with asparagine, glutamine, or alanine resulted in destabilization of adenylate kinase and suggested that this residue is not required for catalysis or nucleotide binding, but is required for the proper tertiary structure of the enzyme. Three possible stabilization roles were proposed for H36, each of which involved charge transfer or hydrogen bonding with other amino acids.

Recently, Thomas et al. (1992) have expressed shortened and full length versions of the rat liver F_1-ATPase β subunit in *E. coli*. One of the shortened genes encoded an asparagine substitution for the conserved histidine. Nucleotide binding assays of the purified product showed that this peptide bound TNP-ATP with the same affinity and stoichiometry as a similarly expressed and purified full length wild type β subunit. This result supports the conclusions of Tian et al. (1988) concerning the lack of function of the conserved histidine in nucleotide binding. Unfortunately, this approach does not address the role of this residue in the context of the entire ATPase complex. Tian et al.(1988) proposed that the primary role of the conserved histidine was stabilization of the tertiary structure of adenylate kinase. If this residue fulfills the same function in the β subunit of F_1, then mutation of this residue might lead to destabilization of the entire complex by distortion of the structure of the three β subunits.

In chapters 2 and 3 of this work the function of the conserved hisitidine residue in the β subunit of the yeast *Saccharomyces cerevisiae* F_0F_1-ATPase was examined by site-directed mutagenesis. This residue, H211, was mutated to asparagine, aspartate, lysine, isoleucine, and alanine in five separate constructs. An additional mutation was constructed by substituting phenylalanine for leucine at position 203. The mutated subunits were expressed in a yeast host strain which produced no β subunit of its own and which could not respire aerobically. Transcription of the mutated genes was under

the control of the natural *ATP2* promoter. Expression of wild type *ATP2* in the host strain allowed aerobic respiration, and F_0F_1-ATPase activity isolated from the resulting strain was indistinguishable from wild type by criteria of kinetic constants and oligomycin sensitivity. Expression of the mutated genes in the host strain resulted in phenotypic and enzyme kinetic changes which allowed several conclusions about the functions of H211 and L203 to be drawn.

H211 and L203 are not Required for Catalysis

Strain βH211N is capable of slow growth on a non-fermentable carbon source, the other position 211 mutants are not. This demonstrates that H211 is not required for catalysis, but is required for the normal functioning of the enzyme. The aerobic repiration of strain βH211N probably owes to its structural similarity to histidine. The potential of the asparaginyl amide nitrogen to form a hydrogen bond may also be significant. The three models proposed by Tian et al. (1988) for H36 function in adenylate kinase all invoke electrostatic interactions involving the number 3 nitrogen of the histidine imidazole ring. This nitrogen is fairly isosteric to the amide nitrogen of glutamine. A useful further experiment to evaluate the role of H211 would be the substitution of glutamine at this position. If the roles of βH211 and adenylate kinase H36 are similar, then this mutation should be less deleterious than H211N.

Strain βL203F shows a reduced capacity for aerobic respiration relative to wild type, but grows faster than βH211N. This result suggests that the hydrophobic character of position 203 is important

for function, thus providing further support for the hydrophobic cleft model of Duncan et al. (1986). Further mutations at this position would provide a clearer picture of the role of L203. The introduction of charged residues such as lysine or glutamate, as well as the introduction of other hydrophobic residues such as isoleucine or valine would be instructive.

H211 and L203 Interact in the Wild type Yeast F_0F_1-ATPase Complex

Figures 2.2 and 2.3 show that the amino acids which correspond to yeast F_1 β subunit residues L203 and H211 are predicted to occur on opposite sides of a hydrophobic cleft in other nucleotide binding proteins. This positioning could allow physical or functional interaction between these two residues. A functional interaction in yeast F_0F_1 is supported by the fact that L203F, as a second site mutation, intragenically suppresses the phenotype of each position 211 mutation. Furthermore, the suppression is expressed kinetically as a decrease in the K_m for ATP hydrolysis by each position 211 mutation when paired with L203F. Detailed explanations of this interaction will be speculative until a high resolution structure of the β subunit is available. These data provide important support for the three dimensional model of Duncan et al. (1986).

H211 and L203 are Required for the Stability and Proper Conformation of the F_1 Complex

ATPases carrying substitutions at positions β203 or β211 were found to be unstable when removed from the mitochondrial inner

membrane. All attempts to purify F_1 from the mutant ATPases failed, and the activities of chloroform extracts of the position 211 mutants decreased in a turnover dependent fashion. On the other hand the activities of submitochondrial particles from the mutant strains were stable for months when stored at -70° C and hours when incubated at 30° C.. This implies structural change in the mutant F_1 moieties which results in their inactivation upon removal from F_0.

The effects of oligomycin and organic solvents on ATPase activity of submitochondrial particles from the mutant strains provide further support for structural perturbation of the F_1 complex. The oligomycin sensitivity of the position 211 mutants was decreased relative to the wild type. This probably reflects decreased affinity of the F_1 complex for OSCP. Dupuis et al. (1985a) have studied the binding of radiolabeled OSCP to beef heart F_1-ATPase. They report three binding sites, one of which they believe to be the site through which OSCP exerts its influence. OSCP has a dissociation constant of 80 nM for this site. Similar experiments performed with the mutant ATPases would provide further information about the structural consequences of the mutations. Such experiments would require the development of new purification techniques because of the present inability to purify mutant F_1.

The effects of ethanol and 2-propanol on ATP hydrolysis by submitochondrial particles from the mutant strains were also suggestive of structural changes. Concentrations of 5-10% of these

solvents resulted in as much as 80% inhibition of ATP hydrolysis (Fig. 2.16). This is consistent with further unfolding of ATPase complexes which have exposed hydrophobic surfaces due to improper folding or structural perturbations.

H211 is required for the Proper Assembly of the F_0F_1-ATPase

Immunological characterization of the mutant F_0F_1-ATPases led to the conclusion that no more than one third as much mutant enzyme is produced as wild type. This could owe to folding problems in the mutant β subunits. Rao et al (1988) have noted that isolated F_1 subunits would not reassemble unless the β subunits bound ATP. If the mutated β subunits have impaired nucleotide binding, then the rate of assembly of F_1 complexes may be negatively affected. Alternatively, improper folding of the β subunits may interfere with recognition by the assembly apparatus. This possibility could be investigated by studies of *in vitro* assembly of the mutated subunits into whole mitochondria. Pulse-chase experiments using radiolabeled subunits would allow comparison to the wild type assembly pathway, this might reveal bottlenecks in assembly caused by the mutations. Immunoprecipitation using antibodies to mitochondrial hsp70 or $hsp60^{MIF4}$ would allow comparisons of the association of the wild type and mutant subunits with these proteins.

H211 is Indirectly Involved in Substrate Binding and is Required for the Proper Conformation of the Active Site.

The K_m values for ATP hydrolysis by the position 211 mutants were determined and found to be greater than wild type. From this

it was concluded that H211 is at least indirectly involved in substrate binding. Such a role could be served by a contribution to active site conformation. This possibility was investigated by determining the pK_a values of the groups involved in catalysis by the wild type enzyme and mutant enzymes from strains βH211K, βH211D, and βH211N. The data obtained for the wild type and mutant ATPases were consistent with an acid-base catalyzed reaction. The mutations had no effect on the pK_a of the acidic group, but resulted in decreases of 1.3-1.7 units in the pK_a of the basic group. This type of effect is suggestive of a microenvironmental effect on the pK_a of the catalytic general base. If this basic group were subjected to a more hydrophobic environment, its pK_a could be expected to shift toward neutrality. Such a microenvironmental effect could be confirmed by the use of fluorescent nucleotide analogs. The emission spectrum of a fluor changes as a function of hydrophobicity. The emission spectra of fluorescent nucleotide analogs bound to the mutant ATPases could be compared to those observed when the same analogs are bound to the wild type enzyme. Comparison of these spectra to those of the nucleotide analogs in media of varying hydrophobicity would allow conclusions to be drawn about the relative hydrophobicities of the mutant and wild type active sites.

In summary, H211 of the yeast F_0F_1-ATPase β subunit fulfills several roles. It is required for the proper assembly and conformation of the F_0F_1 complex, it is required for the stability of the F_1 moiety, it is indirectly involved in substrate binding, and it

contributes to the structure of the active site. To an unknown extent this role is dependent upon an interaction with L203. The substitution of phenylalanine for leucine at position 203 results in decreased ATPase stability with no effect on K_m for ATP hydrolysis. L203 is therefore required for the structural stabilization of the F1 complex, but the substitution of phenylalanine at this position does not appear to effect nucleotide binding. The interaction of L203 and H211 is consistent with a model for the tertiary structure of the β subunit proposed by Duncan et al. (1986). The stabilization role of H211 is also consistent with the role played by the homologous residue in adenylate kinase (Tian et al., 1988). This indirectly supports the model of Duncan et al. which is based on the structure of adenylate kinase.

The final chapter of this work is concerned with the regulatory subunits of beef heart and *E. coli* F_1-ATPases. An expression system for the ε subunit of *E. coli* F_1-ATPase was developed in the laboratory of Gino Van Heeke, and the biological activities of the recombinant products were assayed (Van Heeke et al., submitted). The recombinant ε subunit inhibited *E. coli* ATPase activity noncompetitively with a K_i of 11 nM, but had no effect on beef heart F_1-ATPase. These characteristics are typical of the authentic ε subunit. In addition, nearly identical results were achieved with a fusion construct in which the ε subunit N-terminus was fused to the C-terminus of human carbonic anhydrase isozyme II. This result strongly suggested that the N-terminus of the ε subunit does not

interact with F_1. The recombinant ε subunit did not display pH-dependence or turnover-dependence.

A synthetic gene encoding the beef heart F_1-ATPase inhibitor protein was designed and constructed, and its expression and purification were optimized, in the laboratory of Gino Van Heeke. Wild type recombinant F_1I was shown to have the characteristics of authentic F_1I with respect to K_i, pH-dependence, and turnover-dependence. Recombinant F_1I had no effect on *E. coli* F_1-ATPase. Based on these results it was concluded that *E. coli* ε and beef heart F_1I employ different mechanisms of action regardless of a short segment of sequence homology in a region known to be important to the function of F_1I. In further experiments this segment of F_1I (R35-L45) was analyzed by alanine scanning mutagenesis. The results show that alanine substitutions on the hydrophilic face of a predicted α helix have little or no effect on inhibition by F_1I. Alanine substitutions on the hydrophobic face of the helix interfere to a greater extent, with one mutation (L42A) completely abolishing inhibition. These results were interpreted in terms of the effects on hydrophobic interactions. It is apparent that specific side chains on the hydrophobic face of the helix are critical to inhibition by F_1I, this function is likely to be dependent on specific interaction with hydrophobic residues on the β subunit of F_1. Further experiments have been planned which will evaluate the role of other residues which have been implicated in the pH-dependent behavior of F_1I. The potential role of a coiled coil structure in the activity or

regulation of F_1I will also be examined by site-directed mutagenesis and characterization of the resulting inhibitor proteins.

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BIOGRAPHICAL SKETCH

Richard Schnizer was born on March 4, 1961 in Corpus Christi, TX. His father holds a Ph.D. in organometallic chemistry and his parents encouraged his interest in education in general and science in particular from an early age. Richard attended Franklin & Marshall College in Lancaster, PA and graduated in 1983 with a BA in biology. He then traveled to Lincoln, NE, where he attended the University of Nebraska and worked for Dr. John Osterman. In 1986 he completed his MS degree in biological science with an emphasis in genetics and molecular biology. For the next year and a half he worked as a technician in the laboratory of Dr. Sheldon Schuster at the end of which time it was decided that he would be better off with a Ph.D. In January 1988, Richard began his doctoral studies in biological sciences with Dr. Schuster, and in July of 1989 he migrated with the Schuster laboratory to the University of Florida from which he will receive his Ph.D. in biochemistry and molecular biology. After graduation Richard will remain at the University of Florida as a postdoctoral scientist sponsored by BioNebraska Inc. and will be involved in human gene therapy projects aimed at treating osteoporosis and hepatic encephalopathy.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sheldon M. Schuster, Chairman
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of Biochemistry
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1993

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